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XIX. L'ACIDE LACTIQUE EST-IL UN PRODUIT INTERMEDIAIRE DE LA FERMENTATION ALCOOLIQUE?

PAR ALEXANDRE LEBEDEV.

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(*Received June 28th, 1917.*)

Slator [1906, 1907, 1908], Buchner et Meisenheimer [1909] ont démontré que l'acide lactique n'était pas fermentescible par la levure. Après la découverte de Wieland [1913] que celui se décompose facilement en présence du palladium, preparé d'après son excellente méthode, avec formation de CH_3COCO_2H, CO_2 et CH_3CO_2H, il fallait sans doute s'attendre à ce que le même phénomène se produise par la réductase de la levure en présence du bleu de méthylène. Ce point de vue a été appuyé expérimentalement par Palladin, Sabinin et Lovchinovskaya [1915] et Harden et Norris [1915]. De ma part j'ai démontré [1915][1] que l'acide lactique se décomposait dans ces conditions non seulement en solution neutre, mais aussi, d'une manière encore plus intense, en solution acide, de sorte que, contrairement à l'opinion de Palladin, il y aurait, sans intervention de l'eau, une déshydrogénation directe de CHOH par la réductase de la levure. En absence du bleu de méthylène l'acide lactique se décomposait sans formation de l'aldéhyde acétique, mais avec une formation d'acides, probablement de l'acide acétique en prépondérance; la formation de l'alcool était bien douteuse et même, s'il s'en formait une petite quantité, elle était 3 fois plus faible que celle de CO_2 dégagé [Lebedev, 1915, 1916, pp. 741–3], ce qui a été confirmé plus tard par Palladin et Sabinin [1916]. J'ai montré en outre que la vitesse de la décoloration du bleu de méthylène ne dépendait pas de la quantité de CH_3COH formé et que celle-ci ne faisait qu'une très faible partie de l'acide employé. Or, dans une expérience j'ai obtenu pour 500 mg. de $CH_3CHOHCO_2H$ neutralisé 20 mg. de CH_3COH et dans l'autre—pour 300 mg.—16·3 mg. de CH_3COH en solution acide et 15·9 en solution neutre. Il a fallu s'attendre à

[1] Ce mémoire a paru avant le mémoire de Harden et Norris que je viens de citer.

ce que, lorsque le bleu de méthylène se décolorait complètement, pour 1 mol. d'acide décomposé 1 mol. de CH_3COH se formât, mais on n'a trouvé que 1/3 de cette quantité. Je profite de l'occasion pour dire que les expériences de Lvoff, citées par Palladin, d'après lesquelles "in the process of glucose fermentation for each atom of hydrogen removed with the help of methylene blue, the production both of alcohol and of carbon dioxide is diminished by one molecule" [Palladin et Sabinin, 1916, p. 187], ne se laissent pas reproduire [Lebedev, 1915, pp. 317–8]. Il y a donc tout lieu à croire que Lvoff a obtenu ce résultat à cause de la méthode défectueuse dont il se servait.

Oppenheimer [1914, p. 60] a supposé que l'acide lactique pouvait se décomposer en présence de l'acide pyruvique en C_2H_6O et CO_2:

(1) $CH_3CHOHCO_2H - H_2 = CH_3COCO_2H$.

(2) $CH_3COCO_2H = CH_3COH + CO_2$.

(3) $CH_3COH + H_2 = CH_3CH_2OH$.

Il a constaté seulement la disparition d'une partie de l'acide lactique ajouté au suc de macération, mais il n'a pas dosé l'alcool, dont il supposait la formation en présence de l'acide pyruvique. Pour combler cette lacune, j'ai répété les expériences d'Oppenheimer, sans connaître celles de Palladin, en me servant, au lieu du suc, de la levure séchée à 35° et en dosant l'alcool par la méthode de Nicloux. Voici le résultat obtenu [Lebedev, 1916, pp. 742–3].

1.	Témoin ...	217 mg. CO_2	5.	$CH_3CHOHCO_2H$	287 mg. CO_2
2.	„	223 „	6.	„	287 „
3.	$CH_3COCOOH$	220 „	7.	$CH_3CHOHCO_2H + CH_3COCO_2H$	296 „
4.	„	228 „	8.	„	297 „

1, 2.	Témoin	436 mg. d'alcool
3, 4.	CH_3COCO_2H (20 mg.)	456 „ „
5, 6.	$CH_3CHOHCO_2H$ (432 mg.) ...	436 „ „
7, 8.	$CH_3CHOHCO_2H + CH_3COCO_2H$	476 „ „

Si l'on tient compte de ce que l'acide pyruvique s'est décomposé en alcool et CO_2, on obtient définitivement:

		CO_2	CH_3CH_2OH
I.	Témoin	440 mg.	436 mg.
II.	CH_3COCO_2H	438 „	446 „
III.	$CH_3CHOHCO_2H$	574 „	436 „
IV.	$CH_3CHOHCO_2H + CH_3COCO_2H$	583 „	466 „

Il s'en suit qu'en présence de l'aldéhyde acétique une partie de $CH_3CHOHCO_2H$ s'est oxydée probablement en CH_3COCO_2H, mais cette partie était tout à fait *insignifiante*. Si l'on calcule d'après la quantité de CO_2 dégagé, on trouve qu'il s'est décomposé 71 % d'acide lactique ajouté (chaque fiole contenait 10 g. de levure de Lebedev, 50 cc. d'eau et 0·2 cc.

de toluène); au contraire, d'après la quantité d'alcool on n'en trouve que 9 %. Le rapport $CO_2 : C_2H_6O$ au lieu d'être égal à 1 est égal à 8! Il est à noter qu'à la fin de l'expérience le liquide ne contenait que des traces de CH_3COH, parce que la réaction de Rimini était très faible. Palladin a trouvé que le rapport était égal à 6, mais il a commis une forte erreur de calcul. Le témoin de l'expérience 15 [Palladin et Sabinin, 1916, p. 194] a donné en 62 h. 400 mg. de CO_2 et 417 mg. d'alcool. En tenant compte de cela, on obtient pour les nos. 1, 2 et 3 par soustraction:

	Carbon dioxide		Alcohol	
1. Pyruvic acid	90	137	59	87
2. Lactic acid	47		28	
3. Lactic and pyruvic acids	295		97	

Or, $295 - 137 = 158$ mg. de CO_2 et $97 - 87 = 10$ mg. de C_2H_6O; on aura donc le rapport vrai $158 : 10 = 15{\cdot}8$! 10 mg. d'alcool ne font que 2 % de la quantité entière de celui-ci (514 mg.), c'est à dire qu'ils ne dépassent pas l'erreur de la méthode de Nicloux qui est de 5 % et que, par conséquent, *Palladin n'a pas démontré que l'acide lactique se décomposait en présence de* CH_3COCO_2H *en* CO_2 *et* C_2H_6O. Il est à remarquer en outre qu'il dosait l'alcool en présence d'une quantité considérable de CH_3COH, ce qui fait le résultat obtenu par lui plus incertain encore. Bien que mes propres expériences soient un peu plus favorables à la théorie d'Oppenheimer que celles de Palladin, il serait tout de même trop osé d'en conclure qu'une fermentation alcoolique de l'acide lactique eût lieu. Je puis répéter ici ce que j'ai dit il y a deux ans à propos des expériences de Palladin: "On a donc aucune raison pour regarder l'acide lactique comme un produit intermédiaire de la fermentation alcoolique" [Lebedev, 1915, p. 306].

D'après mes recherches [Lebedev, 1915, pp. 331–2] l'aldéhyde pyruvique est fermentescible par la levure et par le suc de macération, préparés d'après ma méthode, avec une production de C_2H_6O et de CO_2 en proportion presque égale à 1, si la concentration d'aldéhyde ne dépasse pas 1·5 %. En présence du bleu de méthylène *l'aldéhyde acétique* s'en forme, évidemment par les réactions suivantes:

1. $CH_3.CO.CH\begin{matrix}\diagup OH \\ \diagdown OH\end{matrix} - H_2 = CH_3COCOOH.$
2. $CH_3COCOOH = CO_2 + CH_3COH.$

Je ne décrirai ici que deux de mes expériences, les autres étant analogues [Lebedev, 1915].

Exp. 1. On a pris 2 fioles d'Erlenmeyer (A et B, à peu près 250 cc. chacune) et on les a chargées de 10 g. de levure de Lebedev + 0·25 g. de bleu de méthylène. A la fiole A on a ajouté 50 cc. de solution de CH_3COCOH à 1·3 %, à l'autre—50 cc. de solution de $CH_3CHOHCO_2H$ à 2 %. Après les avoir bien bouchées, on les a secoué à la machine pendant 17 h. et on a dosé l'aldéhyde acétique sous forme de *p*-nitrophenylhydrazone. Voici le résultat obtenu.

A. 65 mg. de CH_3COCOH ... 10·6 mg. de CH_3COH.
B. 1000 mg. „ $CH_3CHOHCO_2H$ ... 14·4 mg. „

Exp. 2. Mêmes conditions que dans l'expérience 1, mais on a dosé de l'aldéhyde après 9 h. La fiole A contenait 50 cc. de solution de CH_3COCOH à 2·6 %; la fiole B—50 cc. de solution de CH_3CHOH à 1 %.

A. 130 mg. de CH_3COCOH ... 13·5 mg. de CH_3COH.
B. 500 mg. „ $CH_3CHOHCO_2H$ ... 21·0 mg. „

Si l'on tient compte de la concentration de substances ajoutées, on constate que la formation de l'aldéhyde acétique est plus intense dans le cas de CH_3COCOH que de $CH_3CHOHCO_2H$. Etant donné que dans des processus biochimiques l'acide lactique est formé de l'aldéhyde pyruvique, on pourrait plutôt affirmer que c'est justement celui-ci qui soit un produit intermédiaire, néanmoins, bien que cette hypothèse soit bien séduisante, je ne le crois pas, parce que la fermentation de l'aldéhyde pyruvique est très lente et faible à cause de sa toxicité pour les diastases de la levure.

En connexion avec les faits relatés ici, je me permets d'attirer l'attention sur un autre mémoire [Lebedev, 1916], où j'ai montré que la levure de Lebedev décomposait l'acide malique en CO_2 et $CH_3CHOHCO_2H$ et qu'en présence du bleu de méthylène l'aldéhyde acétique en était formé. J'ai fait la supposition que cette réaction curieuse allait en trois phases:

1. $CO_2H.CHOH.CH_2.CO_2H = CO_2 + CH_3.CHOH.CO_2H$.
2. $CH_3.CHOH.CO_2H + R = CH_3.CO.CO_2H + RH_2$.
3. $CH_3.CO.CO_2H = CH_3.COH + CO_2$.

On pourrait faire une autre supposition, à savoir:

1. $CO_2H.CHOH.CH_2.CO_2H + R = CO_2H.CO.CH_2.CO_2H + RH_2$.
2. $CO_2H.CO.CH_2.CO_2H = 2CO_2 + CH_3.COH$.

Mais ce point de vue me semble moins plausible, parce que l'aldéhyde acétique se forme de l'acide malique plus lentement et en quantité plus petit que de l'acide lactique.

Le fait intéressant établi par Palladin [Palladin et Sabinin, 1916] que l'acide pyruvique en fermentant simultanément avec de l'acide lactique

dégageait sous l'influence de la levure plus de CO_2 que lorsqu'il fermentait seul, est tout à fait analogue à celui constaté par moi et Polonsky pendant la fermentation simultanée du sucre et de l'acide pyruvique. Nous avons dosé non seulement CO_2, mais aussi C_2H_6O et C_2H_4O. Voici le résultat d'une de nos expériences après la soustraction des quantités correspondantes des CO_2, C_2H_6O et C_2H_4O du témoin. La durée—25½ h. à 22°. CO_2 a été expulsé complètement par un courant d'air à la fin de l'expérience.

	CO_2	C_2H_6O	C_2H_4O
1. 400 mg. $C_{12}H_{22}O_{11}$	189 mg.	208 mg.	0·5 mg.
2. 100 mg. CH_3COCO_2H	25 „	8 „	0·7 „
3. 100 mg. $CH_3COCOOH$ + 100 mg. $C_{12}H_{22}O_{11}$	120 „	48 „	0·7 „
4. 100 mg. $C_{12}H_{22}O_{11}$	49 „	48 „	0·5 „
5. 100 mg. CH_3COCO_2H + 400 mg. $C_{12}H_{22}O_{11}$	243 „	208 „	0·9 „

On voit que pendant la fermentation de $C_3H_4O_3$ et du sucre (en concentrations égales) se forme presque la même quantité de C_2H_4O, au contraire, la quantité de C_2H_6O a été 6 fois et de CO_2 2 fois plus petite dans le cas de $C_3H_4O_3$, de sorte qu'il n'y avait aucun parallélisme entre les fermentations de ces deux substances. Prenons la somme des nos. 2 et 4 et 1 et 2 et comparons les aux nos. 3 et 5, nous obtiendrons les nombres pour la fermentation simultanée des deux substances et celle de chaque substance séparément.

	CO_2	C_2H_6O	C_2H_4O
2 et 4 (100 mg. $C_3H_4O_3$; 100 mg. $C_{12}H_{22}O_{11}$)	74 mg.	56 mg.	1·2 mg.
3 (fermentation simultanée)	120 „	48 „	0·7 „
1 et 2 (400 mg. $C_{12}H_{22}O_{11}$; 100 mg. $C_3H_4O_3$)	214 „	216 „	1·2 „
5 (fermentation simultanée)	243 „	208 „	0·9 „

Pendant la fermentation simultanée il s'est formé plus (+) ou moins (−) que séparément.

	CO_2	C_2H_6O	C_2H_4O
100 mg. $C_3H_4O_3$ + 100 mg. $C_{12}H_{22}O_{11}$	+46 mg.	−8 mg.	−0·5 mg.
100 mg. $C_3H_4O_3$ + 400 mg. $C_{12}H_{22}O_{11}$	+29 „	−8 „	−0·3 „

On peut en conclure que la fermentation du sucre et de $C_3H_4O_3$ va autrement, lorsque les deux substances se trouvent ensemble et que le travail de la carboxylase ne dépend pas de celui de la zymase. A ce propos je dois ajouter que la présence de $C_3H_4O_3$ n'influence pas d'une manière sensible l'autofermentation de la levure; ce fait a été aussi établi par moi et Polonsky. Il n'est pas facile pour le moment de résoudre la question aux dépens de quelle substance l'augmentation de la quantité de CO_2 a lieu. Il est possible qu'il y ait des substances qui suscitent ou favorisent la décomposition de l'acide pyruvique même, comme, par exemple, le sucre dans nos expériences ou l'acide lactique dans celles de Palladin

Palladin veut expliquer la différence énorme qui existe entre la fermentation du sucre et de l'acide lactique par une supposition que la levure vivante travaille autrement que la levure tuée. Sans dire de cela que la levure préparée d'après ma méthode bien qu'elle reste vivante [Lebedev, 1912, p. 36] décompose le sucre en présence et sans toluène absolument de la même manière, je n'indiquerai que sur le fait bien connu que la levure tuée (Acetondauerhefe, Hefanol et cet.) et vivante fermente le sucre avec production de CO_2 et de C_2H_6O en proportion CO_2: C_2H_6O qui est égale à unité.

Il ressort clairement de tout ce que je viens d'exposer que l'assertion de Palladin [Palladin et Sabinin, 1916, p. 195], "At any rate, our experiments give, firstly, a scheme which shows in which direction the work must be conducted, and secondly, prove that alcohol does not present the result of decomposition, but the product of reaction of acetaldehyde by removing the hydrogen from one of the intermediate products of decomposition of glucose," non seulement n'est pas démontrée par lui, mais qu'elle est en outre inexacte, parce que les mêmes idées ont été émises par Neuberg [Neuberg and Kerb, 1912, Neuberg, 1913, p. 13], moi [Lebedev and Griaznov, 1912, p. 3270; Lebedev, 1914] et Kostytschev [1912].

Palladin pense que la réaction de Cannizzaro n'a pas lieu dans la cellule de levure vivante, mais c'est le contraire qui a été montré par Kostytschev [1914]. J'ai démontré en collaboration avec Polonsky que l'aldéhyde acétique qui se formait pendant la fermentation des acides pyruvique et glycérique par la levure de Lebedev se transformait en partie d'après cette réaction en acide acétique et alcool. Cette transformation d'ailleurs ne devient considérable que si les acides sont employés sous forme de leurs sels alcalins.

Voici le résultat de 4 expériences pour $C_2H_4O_2$ et C_2H_4O.

	Acide acétique		Ald. acétique	
Solut. acide	33·3 mg.	33·0 mg.	10·4 mg.	10·0 mg.
Solut. neutre	40·8 „	47·4 „	0·8 „	2·2 „
Témoin	22·6 „	29·4 „	0·2 „	0·2 „

Ou après la soustraction des nombres correspondants au témoin (moyen):

Solut. acide	7·1 mg.	acide acét.	9·9 mg.
Solut. neutre	18·1 „		1·5 „

ald. acét.

Je donne encore le résultat de 5 expériences pour l'alcool et l'acide acétique (après la soustraction):

	Alcool en mg.		Acide acétique en mg.	
Sol. neutre	32; 34; 32	32·7	24·9; 18·2; 18·0	20·4
Sol. acide	16; 8	12	10·7; 3·6	7·2
		moyen		moyen

Nous trouvons donc de l'aldéhyde acétique sous forme de

	C_2H_6O	et	$C_2H_4O_2$
en milieu acide	8 mg.		3·2 mg.
„ neutre	31 mg.		13·6 mg.

On voit de ces chiffres qu'en milieu neutre *la réaction de Cannizzaro va parallèlement avec une réduction de l'aldéhyde acétique par la réductase de levure.* En milieu acide la réaction de Cannizzaro est de beaucoup plus faible.

Nous avons en outre constaté que, si l'on songe d'après la quantité de CO_2 dégagé, la décomposition de l'acide pyruvique en milieu acide est 2 fois plus forte que dans le milieu neutre[1], mais l'excédant de CO_2 n'est pas balancé par les produits nommés plus haut, c'est à dire C_2H_6O, C_2H_4O et $C_2H_4O_2$, de sorte qu'il faut admettre qu'une oxydation directe ou une formation des produits d'une condensation ait lieu. Voici le résultat de 3 expériences.

		m. neutre	m. acide		m. neutre	m. acide
1.	$C_2H_6O + C_2H_4O + C_2H_4O_2$	49·6 mg.	25·5 mg.	CO_2	43·0 mg.	51·0 mg.
2.	„ „ „	37·1 „	32·9 „	„	22·0 „	54·5 „
3.	„ „ „	34·5 „	30·9 „	„	51·0 „	77·0 „
		121·2 „	89·3 „		116 „	182·5 „

Le moyen de 3 expériences est alors:

Milieu neutre 40 mg. $[C_2H_6O + C_2H_4O + C_2H_4O_2]$ correspondant aux 38·7 mg. de CO_2.
„ acide 29·8 „ „ „ „ 60·8 „ „

Nous rappelons ici le fait bien connu que la fermentation du sucre se produit toujours en milieu acide (si l'on ne le fait pas artificiellement neutre ou alcalin); si l'acide pyruvique était un produit intermédiaire, la proportion $CO_2 : [C_2H_6O + C_2H_4O + C_2H_4O_2]$ devrait être au moins égale à 1, mais elle est, comme nous venons de le montrer, considérablement plus grande. C'est pourquoi on ne serait peut-être pas trop étonné, si un jour on trouvait que l'acide pyruvique ne soit pas un produit intermédiaire de la fermentation alcoolique.

Il y a quelques années j'ai fait une observation curieuse que l'acide glycérique se décomposait en présence de la levure de Lebedev d'après l'équation suivante:

$$CH_2OH.CHOH.CO_2H = C_2H_4O + CO_2 + H_2O.$$

Or, je puis à présent affirmer qu'il peut se former pendant la fermentation de cet acide plus d'aldéhyde acétique que dans le cas de l'acide pyruvique et que la décomposition de l'acide glycérique même en milieu acide suit de plus près l'équation donnée plus haut que celle de l'acide pyruvique. Je ferai connaître ici les chiffres moyens de deux expériences faites par moi et Polonsky.

[1] Il va de soi qu'à la fin de l'expérience quelques gouttes d'acide sulfurique ont été ajoutées.

Pour 25 cc. d'eau et 5 g. de levure on a pris 230 mg. d'acide glycérique et 250 mg. d'acide pyruvique. T. = 20°. Les fioles qui servaient au dosage de CO_2 ont été à la fin des expériences chauffées pendant une $\frac{1}{2}$ h. à 70°, après quoi on a fait passer pendant 1 h. un courant d'air à travers le liquide et la soupape de fermentation de Meissl. Les fioles qui servaient pour le dosage des $C_2H_4O_2$ et C_2H_4O ont été bien bouchées. On a dosé l'alcool par la méthode de Nicloux [1906] et le C_2H_4O par la méthode de Ripper [1900]. Voici le résultat obtenu:

	CO_2	C_2H_6O	C_2H_4O	$C_2H_4O_2$
1. Témoin	80	82·5	0·11	11·70
2. Ac. glycér.	124	105·0	10·44	12·15
3. Ac. pyruv.	154	116·0	4·35	15·15

Après la soustraction des chiffres-témoins on a calculé la quantité de C_2H_4O qui correspond à celle des C_2H_6O et C_2H_4O. On trouve par conséquent 32·2 mg. de C_2H_4O + 44 mg. de CO_2 pour l'acide glycérique et 38·8 mg. de C_2H_4O + 74 mg. de CO_2 pour l'acide pyruvique. (On obtient le même résultat en présence et absence de toluène.)

Ce fait dit en faveur de la supposition qu'à cause de la formation successive de l'acide pyruvique, celui-ci soit fermenté dès qu'il se forme, sans donner lieu à la production et l'accumulation des substances secondaires, mais il y a d'autres difficultés, dont j'ai parlé dans le travail cité plus haut qui ne se laissent pas expliquer si facilement et qui restent encore à surmonter pour croire que l'acide pyruvique soit vraiment un produit intermédiaire; comme je viens de montrer dans le mémoire présent, les difficultés deviennent même énormes, si l'on veut admettre que l'acide lactique soit aussi un produit intermédiaire de la fermentation alcoolique, de sorte que je ne crois pas qu'on ne puisse jamais les surmonter.

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XX. CONTRIBUTIONS TO THE BIOCHEMISTRY OF PATHOGENIC ANAEROBES. II. THE ACID PRODUCTION OF *BACILLUS WELCHII* (*B. PERFRINGENS*) AND *BACILLUS SPOROGENES* (METCHNIKOFF).

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In a recent paper, one of us with Harris [Wolf and Harris, 1917] has shown that, in the fermentation with *Bacillus Welchii* (*B. perfringens*), all activity ceases when the reaction of the medium reaches a certain hydrogen ion concentration. The cessation of growth appeared to be connected with the production of acids. We were able to confirm the statements of previous observers, who have worked on acid production, that normal butyric acid was one of the constituents of the fermentation liquid, by isolating the acid in a fairly pure condition and identifying it. Other acids were also present.

With the *B. sporogenes* of Metchnikoff, there was also a large amount of acid formed when the organisms acted on various media. In milk it was difficult to say from what component of the medium the acid arose.

Owing to the difficulties in titrating a complex medium, containing buffer salts and amphoteric nitrogenous compounds, it was impossible to ascertain what were the amounts of acids that these bacteria produced.

As the production of acids by these two organisms has been advanced to account for certain clinical features in gas gangrene infections [Wright, 1917], it appeared advisable to devote some attention to an investigation of this property.

For some years the only method of estimating quantitatively the volatile acid production of micro-organisms has been that of Duclaux [1900]. Owing

to certain inherent difficulties in the method it has not been very largely used by biochemists. Recently there appeared an article by Dyer [1917], in which he described a new method of dealing with the volatile fatty acids in mixtures and suggested that it might be employed in the study of acid production in bacteriological media. Briefly stated, this method consists of a determination of the distilling rate in steam of an acid solution when the latter is maintained at constant volume. The distillate is collected in portions and titrated. The number of cc. of alkali required to neutralise each portion is noted and the percentage of acid calculated. The amount of acid in a given fraction, the "Distilling Constant" for the fraction, is plotted against the corresponding amount of the distillate on logarithmic coordinate paper. Pure acids are graphically represented as straight lines. With mixtures of acids, curves are obtained, the nature of which depends on the acid components.

With a mixture of any two volatile acids, the first part of the curve which is obtained occupies a position intermediate to the lines representing the distilling rate of the higher and lower boiling acids. As the higher boiling acid is removed, the curve gradually becomes parallel with the line representing the lower boiling acid. This serves to identify the lower acid with certainty. Dyer showed that it was possible to ascertain the amount of each acid present when the distilling rate of the mixture had been determined, and the acids present were known. He also gave a number of chemical tests to enable one to identify the acids present in the distillates.

The method appeared to present obvious advantages for the work in which we were engaged, and we therefore made a certain number of preliminary experiments before using it on fermentation mixtures.

Our apparatus differed in one essential from that of Dyer, in that the heating of both flasks was done by gas instead of electricity. By using von Babo's flask guards underneath the distilling and steam generating flasks, and especially by insulating heavily all connecting tubes throughout the apparatus, it was possible to regulate the heating flame, so that the volume of the acid mixture kept constant through a long distillation.

Our first experiments were made with pure acids. The acids chosen were those that are known to be present in fermentation liquors, and ranged from formic to caproic acids.

The distilling rates of these acids were then graphically represented on a logarithmic chart, and the lines which were obtained were used to interpret the results of other distillation experiments. The distilling rates found were very similar to those obtained by Dyer.

Dyer used his method to identify the lower boiling acid in any given mixture by means of the parallelism of the curve representing the last portion of his distillate to a known curve. He identified the higher boiling acid by means of chemical tests. Working on artificial mixtures of known acids, this is satisfactory. With an unknown mixture of several acids resulting from a fermentation experiment, this does not apply. We were therefore compelled to modify Dyer's method in a manner which made it much more tedious, but ultimately allowed us to arrive at more satisfactory results.

The reasons for making this change were based on the following considerations. When a mixture of two acids is distilled the nature of the curve obtained will depend on the distilling rate of each component. With a mixture containing an acid high in the series, for example, caproic acid, the first part of the curve will approximate to the straight line on the chart which represents caproic acid, and the degree of this approximation will depend on the relative amount of that acid present.

The final part of the curve will become parallel to the straight line on the chart which represents the lower acid.

If present in small amount the higher acid can be concentrated and partially separated from the lower acid by fractionation so that the curve obtained on redistillation will approximate so closely to the acid line for caproic acid that it can thereby be identified.

That is, we applied Dyer's method to determine the nature of fractionated portions of the fermentation liquid; and since the amount of acid in each of these portions was known, the calculation of the separate acids in the fermentation liquid could be made. Using caproic acid and butyric acid in a mixture, by means of this method of fractionation it was possible to obtain two portions of the mixture, one containing a relatively large amount of caproic acid and the other consisting chiefly of butyric acid. When distilled separately the curves obtained approximated to the lines for caproic acid and butyric acid on the chart.

When more than two acids are present the problem of identifying and estimating them becomes complicated. The difficulty is always with the acids of intermediate boiling point and appears in any method of separation of the acid series. In the Duclaux method this is emphasised in a recent criticism by Upson, Plum and Schott [1917]. In our opinion, Dyer's method affords no solution of this problem of dealing with the intermediate acids of a mixture. In conjunction with it we have had to resort to a process of fractionation which we describe below.

For example, in a mixture containing acetic, butyric, valeric and caproic acids, it is impossible without a number of refractionations, which is beyond the scope of ordinary technique, to separate these acids so that the colour tests for each will be distinctive. Moreover, the colour tests, which are undoubtedly helpful in determining what acids are present, are not specific; and unless the acid is present in sufficient quantity a positive result will not be obtained.

In making a quantitative analysis of a fermentation liquid we found it necessary to proceed in the following way:

(1) To distil a portion exhaustively in order to find the total amount of volatile acid present.

(2) To distil the fermented liquid as a simple acid mixture, according to Dyer's method, and graphically represent the distilling rate on a logarithmic chart. The curve obtained indicates the lowest acid and the probable nature of the highest acid in the liquid.

(3) To collect fractionated portions of a series of separate steam distillations conducted under conditions identical with the above. The total amount of acid in these portions can be calculated from the previously ascertained distilling rate of the mixture.

The application of chemical tests to the concentrated distillates of these fractions affords useful information at this stage.

(4) Finally, from consideration of the data so obtained to prepare an artificial mixture which will show a distilling rate sufficiently close to that of the fermentation liquid to warrant the assumption that they correspond in composition.

As will be seen from the above, the method is tedious in application, but it certainly gives important quantitative information regarding the acids produced by various organisms. At the same time it must be emphasised that the process above described will afford only approximate results.

After a large amount of preliminary work on mixtures of pure acids from which the limit of accuracy of the method was ascertained, we proceeded to examine some of the fermentation liquids yielded by *B. perfringens* and *B. sporogenes*.

The organisms were grown in vacuo in a Winchester quart bottle in various media.

The apparatus used for incubation was that employed by Wolf and Harris [1917]. Usually 1500 cc. of culture medium was employed, and samples taken from time to time. In all instances the production of gas was followed to correlate gas production with amount of acid produced.

Fermentation No. 1.

Bacillus sporogenes (*Metchnikoff*) *on milk.*

100 cc. of the sample of liquid were taken from the fermentation flask under aseptic precautions, placed in the distilling flask and rendered acid to Congo red paper by the addition of normal sulphuric acid.

The volume was then made up to 150 cc. and the flask connected to the condenser. The burner employed to heat the flask was placed in position, and at the first appearance of bubbles in the liquid, the gas jet was lowered so as to give a definite size of flame. The steam generating flask which contained water actively boiling was immediately connected to the distilling flask and the distillation begun. Fractions of 10 cc. were collected up to 50 cc. Then successively larger fractions of 50 cc., 100 cc., 200 cc., and 500 cc. were collected. It was usually found unnecessary to continue the distillation beyond 500 cc. in order to obtain sufficient data for the acid curve.

The total amount of acid present was obtained by exhaustively distilling another portion of the original liquid in a separate apparatus, and titrating the distillate with decinormal sodium hydrate. In all these experiments phenolphthalein was the indicator used.

Table I. *Fermentation at the end of* 117 *hours.*

100 cc. liquid = 47·6 cc. $N/10$ sodium hydrate.

Fraction of dist. in cc. ...	10	20	30	40	50	100	200	400	500
% acid in fraction ...	15·3	26·0	34·6	41·9	47·6	64·6	76·5	87·2	91·0

The figures in the lower column denoting the "Distilling Constants" were plotted on the chart against the corresponding fraction of the distillate.

From a consideration of the curve obtained (Fig. 1), it will be seen that the fermentation liquid contains a mixture of higher and lower volatile fatty acids, and that the curve tends to run parallel with the curve for acetic acid, after 75 % has distilled over.

The "distilling constant" for the first 10 cc. fraction (15·3) is very high when the presence of acetic acid in the mixture is taken into account, and caproic acid or higher acids were therefore suspected.

On applying Dyer's colour tests to the first portions of the distillate the presence of caproic acid was confirmed. The process of fractionating the fermentation liquid was therefore next employed in the following manner.

Fractional distillation.

100 cc. of the liquid were acidified with decinormal sulphuric acid and the volume made up to 150 cc. This was then steam distilled under the usual conditions of constant volume.

The first 25 cc. were collected and labelled "*A*." The intermediate portion distilling over between 25 cc. and 75 cc. labelled "*B*"; and the third portion distilling over between 75 cc. and 175 cc. labelled "*C*."

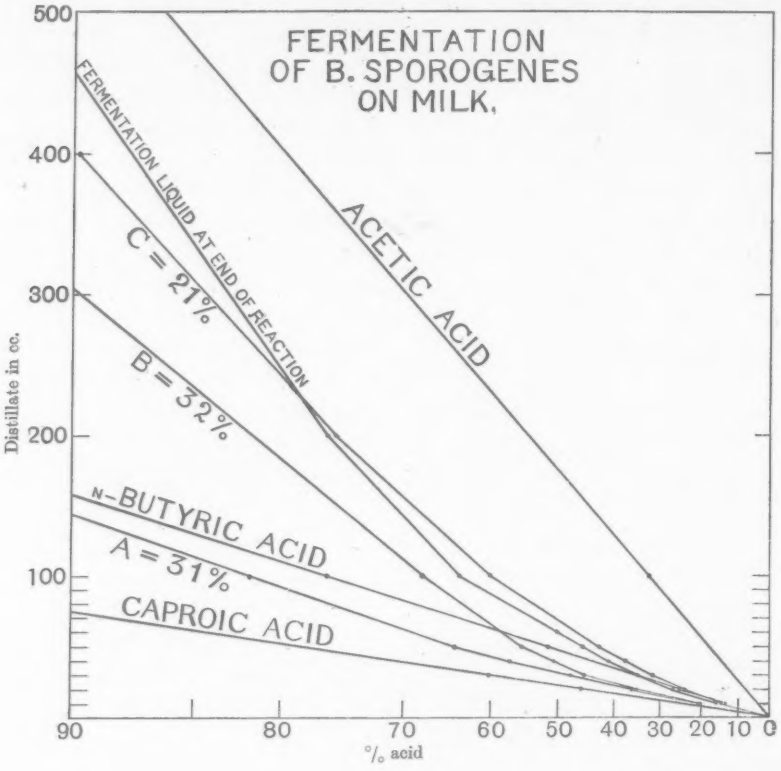

Fig. 1

This was repeated with other three portions of 100 cc. of the fermentation liquid. The four "*A*" fractions of 25 cc. were then mixed, an aliquot part drawn off and titrated; and the remainder placed in the distilling flask and the volume made up to 150 cc. This was steam distilled. The results were as follows:

TABLE II. *"A" portion = 31 % of total acid.*

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	20·5	36·0	47·6	57·1	64·4	83·3	92·8	95·7

On examining this curve ("*A*" Fig. 1), it will be seen that it runs very nearly parallel with the line of *n*-butyric acid after 65 % of the total acid has distilled over.

The very high distilling constant for the first 10 cc. indicates that caproic acid was probably present, since it approximates to the "distilling constant" for that acid which is 22·6 %.

After concentrating the distillates caproic acid was definitely proved to be present by the colour tests. In this portion there must have been an almost complete absence of acids lower in the series than butyric acid, so that the 31 % of the acid of the fermentation liquid consisted almost entirely of acids high in the series and of these caproic and butyric acids were two.

The intermediate portion "*B*" was dealt with in a similar way, but since the mixed fraction constituting this portion amounted to 400 cc. it was evaporated on the water-bath after neutralisation with decinormal sodium hydrate and made up to 100 cc. exactly. 25 cc. of this solution were put in a separate apparatus, acidified with normal sulphuric acid to liberate the acids and exhaustively distilled. From the titration of this distillate the total amount of acid in the remaining 75 cc. was calculated.

The remaining 75 cc. were then acidified with normal sulphuric acid, made up to 150 cc. and steam distilled.

TABLE III. *"B" portion = 32 % of total acid.*

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	20·4	35·6	45·0	50·4	55·4	68·0	81·7	93·0

The very high "distilling constant" (20·4) obtained for the first fraction indicates that caproic or higher acids were also present in this fraction. The curve ("*B*" Fig. 1) takes a very decided bend near the point when 55 % of the acids have distilled over indicating that these are of a mixed nature. With ferric chloride and ether a test corresponding to valeric acid was given, but the colour test for caproic acid was not positive.

This fraction, amounting to 32 % of the total acid of the fermentation liquid, consisted then of valeric acid as well as of lower acids. The amount of caproic acid, which was undoubtedly present, was evidently too small to give a positive colour test.

The third portion "*C*" was dealt with in exactly the same way as the

preceding. It corresponded to 21 % of the total acid in the fermentation liquid.

The results of the distillation were as follows:

TABLE IV. *"C" portion = 21 % of total acid.*

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	14·9	24·0	31·6	37·3	42·5	59·9	76·0	89·8

When the distillates from this portion were concentrated and the ether-ferric chloride test applied none of the precipitated iron salt was found to be soluble in the ether solution, indicating the absence in quantity of acids higher than butyric in this portion. The last part of the curve ("*C*" Fig. 1) was almost parallel with the normal curve for acetic acid. Formic acid was never detected in any portion of the distillate.

This fraction, corresponding to 21 % of the total acid in the fermentation liquid, must have had butyric acid as its chief constituent along with a certain amount of acetic acid.

An analysis of the above results leads to the following conclusions:

(1) Caproic, valeric, butyric and acetic acids were present in the fermentation liquid since these have been demonstrated in the various fractions.

(2) From "*A*" portion (Table II), that 31 % of the acid of the fermentation liquid consisted almost wholly of butyric, valeric, caproic and higher acids.

(3) In the "*B*" portion (Table III), which corresponded to 32 % of the total acids in the fermentation liquid, about one-half consisted of butyric and valeric acids.

(4) Acetic acid was present in considerable quantity both in the "*B*" and "*C*" portion.

(5) Formic acid was absent.

It was impossible to estimate with any degree of accuracy the amount of these acids present. Nor could the presence or absence of propionic acid be definitely established either by colour tests or by consideration of the acid curves obtained.

The remaining quantity of acid which had not been dealt with as a fraction was equivalent to 16 % of the total acids in the fermentation liquid. It must have consisted chiefly of lower acids, probably acetic acid.

The problem then was to determine in what proportion the various acids were present. After some consideration it was decided that an attempt might be made to arrive at a solution by distilling artificial mixtures of caproic, valeric, butyric and acetic acids the result of which would give a close approximation to the figures obtained in distilling the fermentation liquid.

In making up such a mixture the calculation of the relative quantities of the components to be chosen was based on the rough estimate of the composition of the fermentation liquid which has just been made.

After several trials the following mixture was distilled and the results tabulated:

Acetic acid	25·2 %
Butyric ,,	17·2
Valeric ,,	16·2
Caproic ,,	41·4

TABLE V.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	14·9	25·6	34·1	41·4	47·6	65·2	77·5	87·7	90·0

The distilling rate of this mixture is an approximation to that of the fermentation liquid, as will be seen when the two tables are compared. It is clear however that the composition of the artificial mixture cannot be regarded as any more than this. No account has been taken of the presence or absence of propionic acid, or of small quantities of acid higher in the series than caproic acid; and it was found that the amount of intermediate acids could be varied considerably without appreciably changing the distilling rate of the mixture.

FERMENTATION No. 2.

B. perfringens *on milk.*

The fermentation liquid obtained by the growth of *B. perfringens* on milk under strict anaerobic conditions was subjected to a similar examination. As before, the sample was drawn off at the end of the fermentation when no further gas was evolved, and a quantity distilled exhaustively to determine the total amount of volatile acids present. 100 cc. of the liquid were then steam distilled under the usual conditions, the distilling rate of the acid mixture determined, and the results plotted on the logarithmic chart.

TABLE VI.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	8·4	15·8	22·3	28·2	33·3	52·3	71·3	86·6	91·3

From a consideration of the curve (Fig. 2) it will be seen that 52·3 % of the whole distills over in 100 cc., suggesting that butyric acid and some lower acids were present in the mixture. The presence of valeric acid and caproic acid could not be detected by colour tests when these were made, using the

first portion of the distillate, and these acids could only have been present in small quantities.

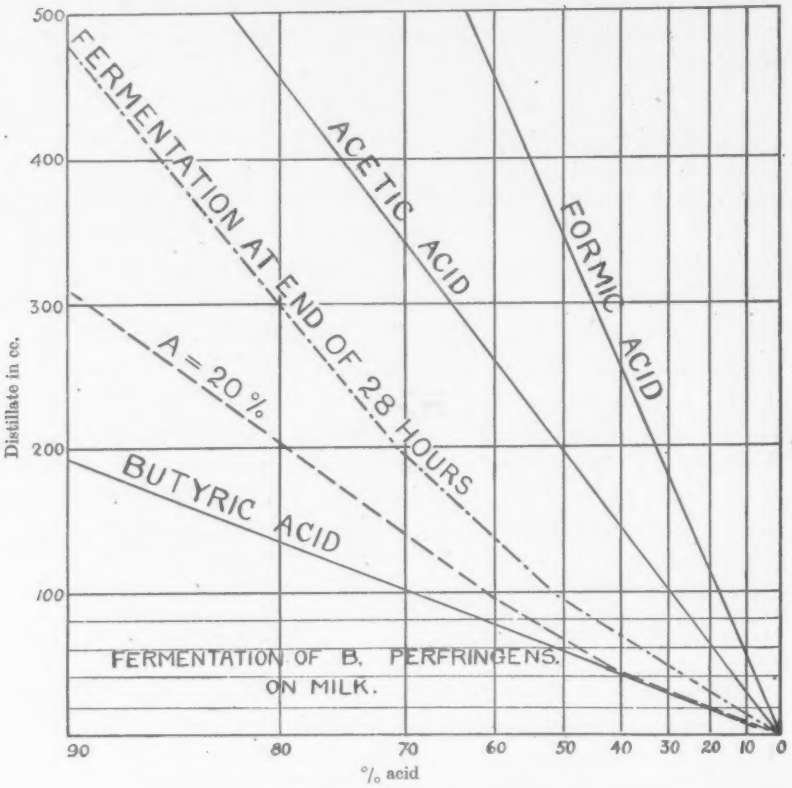

Fig. 2

Fractional distillation.

Three portions of 100 cc. each of the fermentation liquid were acidified with normal sulphuric acid and the volumes made up to 150 cc. and steam distilled. The first fractions of 25 cc. were collected and mixed. After titration of an aliquot part the remainder was steam distilled. The quantity of acid used represented 20 % of the total acid present in the fermentation liquid.

TABLE VII.

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	12·3	21·7	29·6	36·8	43·3	61·6	79·6	97·4

Acids higher in the series than butyric could not be detected by colour tests in this portion which probably consisted of butyric and lower acids. This is evident from the nature of the curve which finally becomes parallel to that of acetic acid. Also the "distilling constant" for the first 10 cc. (12·3) is considerably higher than that of propionic acid. Since valeric and higher acids are not present in any great quantity, butyric acid only could account for this fairly high figure. It was unnecessary to consider further fractionation since the assumption was that butyric and acetic acids constituted this mixture.

Assuming that the fermentation liquid consisted chiefly of butyric acid and acetic acid, it should be possible to find out what amount of each was present from its "distilling constant" by means of a simple calculation.

The "distilling constant" for 50 cc. of the fermentation liquid containing the mixed acids ... ... = 33·3 %
"Distilling constant" of butyric acid ... ... = 45·6
"Distilling constant" of acetic acid ... ... = 16·5

So that such a mixture would correspond approximately to 42 % acetic acid and 58 % butyric acid.

A mixture containing 40 % acetic acid and 60 % butyric acid was distilled under the usual conditions with the following results:

TABLE VIII. *Total acid* = 63·9 *cc. N*/10 *sodium hydrate.*

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	8·6	15·8	22·4	28·5	33·8	53·5	73·9	89·0	92·5

When this table is compared with Table VI it will be seen that the fermentation liquid obtained from the action of *B. perfringens* on milk consisted approximately of the volatile fatty acids, butyric and acetic, in the proportions distilled.

It is of course open to objection that no account has been taken of the possible presence of propionic acid or of the acids higher in the series.

The colour test for propionic acid which is given by Dyer is perhaps the least definite of all; and since this acid occupies a position intermediate to butyric and acetic acids an attempt at isolation would entail a long and careful fractional distillation.

The artificial mixture of butyric and acetic acids can therefore only be regarded as an approximation to the composition of the fermentation liquid.

FERMENTATION No. 3.

B. sporogenes *on* 2 % *glucose peptone.*

100 cc. of the liquid at the end of fermentation was placed in the distilling flask, rendered acid to Congo red with normal sulphuric acid and the volume made up to 150 cc.

This was steam distilled under constant volume and the fractions collected and titrated as before.

The results given in Table IX were plotted on the chart. The total amount of acid present was obtained by distilling a separate 100 cc. exhaustively.

TABLE IX.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	7·2	13·2	18·7	23·8	28·5	44·9	65·5	86·1	92·4

From a consideration of the curve obtained it was seen that the final part tended to become parallel with the line for acetic acid. The "distilling constant" for the first 10 cc. fraction is 7·2, that for the first 10 cc. of a solution of pure propionic acid is 7·03. But since acetic acid is present in the mixture, the "distilling constant" of which is 3·5, an acid with a much higher distilling rate than propionic acid must be present.

Colour tests were applied to the collected distillates and also to fractions which contained the higher acids in greater proportion. In none of these was the presence of valeric acid or acids higher in the series shown.

The acid associated with acetic acid in the fermentation liquid must then have been butyric acid, the "distilling constant" for the first 10 cc. of which is 11·47 %.

In order to confirm the presence of this acid the fermentation liquid was subjected to a fractional distillation as before, the higher acids being obtained in more concentrated form. The following table shows the results obtained from the redistillation.

TABLE X. *Fraction* = 15 % *of total acid.*

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	10·7	19·8	27·7	34·4	39·9	58·9	77·2	91·5	95·4

The first 10 cc. gives a "distilling constant" of 10·7 %. This indicates conclusively the presence of butyric acid in the fermentation liquid.

Assuming that butyric and acetic acids alone were present, a mixture containing these in pure form was made up and redistilled. The proportion in which they were mixed was obtained, as in the previous case, by a calcula-

tion based on the distilling rates of the fermentation liquid, pure acetic acid and pure butyric acid.

The "distilling constant" for the first 50 cc. of the fermentation liquid ... ... = 28·5 %

"Distilling constant" of butyric acid ... ... = 45·6

"Distilling constant" of acetic acid ... ... = 16·5

To obtain a mixture of butyric and acetic acids in which the first 50 cc. shall contain 28·5 % of the total acid, these acids must be present in the following proportion:

Butyric acid = 41 %

Acetic ,, = 59

Such a mixture when distilled gave the following results:

TABLE XI.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	7·0	13·3	18·9	23·8	28·3	45·5	64·6	84·6	90·9

Comparing this table with the results obtained from the distillation of the fermentation liquid shown in Table IX it will be observed that the latter must be similar in composition to the artificial mixture.

As before, no account has been taken of the presence or absence of propionic acid in the fermentation liquid, because it was impossible by colour tests or a consideration of the curve to determine its presence or absence. The close approximation of the curve of the above artificial mixture to that of the fermentation liquid cannot be taken as proof that the fermentation liquid contained no propionic acid. By the introduction of a certain quantity of propionic acid into a mixture of butyric and acetic acids a curve almost identical with that of acetic and butyric acids could be obtained.

The following example will illustrate this:

A mixture of butyric and acetic acids in almost equal parts was distilled under the usual conditions, and the distilling rate obtained.

The same mixture of these two acids was taken and 20 % of propionic acid added. This second combination was distilled under the same conditions as the first.

The results showing distilling rates of the two mixtures are tabulated for comparison.

TABLE XII.

Fraction in cc.	10	20	30	40	50	100	200	400	500
Butyric and acetic acid	8·0	14·9	21·1	26·6	31·7	51·2	73·4	91·0	95·0
Butyric and acetic acid with 20 % propionic	7·7	14·5	20·5	26·1	31·2	51·0	73·1	90·3	94·1

Here we have two acid mixtures differing widely in composition yet giving almost identical distilling rates.

This experiment could be repeated with a combination of any three other consecutive acids in the series, so as to demonstrate generally that the introduction of the "intermediate" acid in moderate amount has little effect on the distilling rates of the combination of the other two.

There appears to be no way out of this difficulty of dealing with the "intermediate" acid except by a long and tedious fractionation.

Fermentation No. 4.

B. perfringens *on* 2 % *glucose peptone.*

100 cc. of the sample at the end of fermentation was taken, acidified with normal sulphuric acid till acid to Congo red, the volume made up to 150 cc. and distilled as before.

The following results were obtained:

Table XIII.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	7·4	13·3	18·1	22·5	26·7	43·7	65·0	87·6	93·5

Comparing this with the results of the previous experiment, it will be seen that the volatile fatty acids produced by the action of *B. perfringens* and *B. sporogenes* on 2 % glucose peptone are almost identical. Also the total quantity of volatile acid formed by each organism was found to be the same. This fact is interesting when the great difference in the nature of the acid production by the growth of the organisms on milk is considered. Large quantities of valeric and caproic acids result from the action of *B. sporogenes* on the latter medium and this distinguishes this organism at once from *B. perfringens* which apparently produces very small amounts of these higher acids. A single distillation of a fermented milk would always serve to differentiate the two, but this would be impossible if the medium were glucose peptone.

On reviewing the above experiments we concluded that Dyer's method may be employed with limitations in the study of volatile acid production of fermented media.

It is impossible by a single distillation alone to determine from the distilling rates of the mixture the nature of the acids present. Partial isolation of the constituents is necessary to establish both the nature and the amount of each acid present. Dyer's colour tests are only of value when the acid exists in an almost pure state.

By employing the method of partial fractionation which has been described, an approximate analysis of the volatile acids present may be made. As we have shown, exact confirmation of such an analysis cannot be obtained by identifying the distilling rate of the fermentation liquid with that of an artificial mixture. As a means of obtaining comparative results the scheme of analysis which has been outlined above is decidedly useful.

When the distilling rates of a fairly large number of artificial mixtures have been graphically represented on a chart, the approximate composition of a fermented medium can at once be obtained simply by identifying the mixture with which its distilling rate coincides.

Non-volatile acid production.

The acidity of these fermentation liquors was found to be due partly to the formation of a relatively large quantity of non-volatile acids. The nature of these acids is not discussed in this paper. Their estimation was made solely to demonstrate that they cannot be neglected in any consideration of the effects of acid production. The following method was employed for their determination.

100 cc. of the fermentation liquid was acidified with normal sulphuric acid and exhaustively steam distilled. The distillate was titrated with decinormal sodium hydrate, using phenolphthalein as an indicator, and the total amount of volatile acids present ascertained.

The residue in the distilling flask, containing the non-volatile portion, was transferred to a liquid extraction apparatus and the acids dissolved out with ether. This operation was a continuous one, and extended over a period of forty-eight hours.

The ethereal solution was evaporated to dryness and the residue dissolved in water. The solution containing the acids was then filtered and titrated with decinormal sodium hydrate.

The following table shows the results obtained in the examination of the fermentation liquors which were produced by the action of *B. sporogenes* and *B. perfringens* on milk and 2 % glucose peptone.

Organism	Medium	Relative vol. acidity	Relative non-vol. acidity
B. sporogenes	Milk	69 %	31 %
	2 % glucose peptone	55	45
B. perfringens	Milk	68·5	31·5
	2 % glucose peptone	50	50

SUMMARY.

The investigation of the acid production of our fermentations of *Bacillus perfringens* and *Bacillus sporogenes* shows that large quantities of volatile acids are produced. Of these butyric acid may be said to be a constant component, but it is worthy of note that in the action of *B. sporogenes* on milk, acids higher in the series than butyric are formed in considerable quantity. The action of *B. sporogenes* on milk results in the production of caproic and valeric acids. The presence of these higher acids is not however a specific quality of *B. sporogenes*, for the fermentation of glucose peptone by this organism produces only acetic and butyric acids. We have been unable to detect propionic acid in any of the fermentation liquids, but its presence is not excluded. Formic acid was not present.

In addition to volatile acids, there is a notable proportion of non-volatile acids, amounting roughly to 40 % of the total acid production. What the nature of these acids is has not yet been ascertained.

An experimental critique of the Dyer method of estimating volatile fatty acids has been made. This method, while perfectly satisfactory in the form stated by its author in dealing with a mixture of two volatile acids, the nature of which is known, fails when a mixture of unknown acids has to be analysed. The colour tests proposed by him are satisfactory when dealing with pure acids, but are not as positive as could be desired for the identification of an acid in the mixture. The separation of the acids is necessary before any reliance can be placed on these colour tests.

By refractionation clearer results are obtained; but when a mixture of more than two acids is dealt with, the components of which are consecutive in the acid series, the task of identifying and estimating them is a problem of extreme difficulty.

To what extent Dyer's method is applicable for this purpose has been indicated in the foregoing paper.

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XXI. CONTRIBUTIONS TO THE BIOCHEMISTRY OF PATHOGENIC ANAEROBES. III. THE EFFECT OF ACIDS ON THE GROWTH OF *BACILLUS WELCHII* (*B. PERFRINGENS*) AND *BACILLUS SPOROGENES* (METCHNIKOFF)

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In our first paper [1917] on the growth of pathogenic anaerobes we showed that growth of the bacillus of Welch ceased at a very definite hydrogen ion concentration when the organism was inoculated into milk. We also referred to the possibility of the inhibiting action of acids having a certain clinical interest in the treatment of infection by these bacteria.

The cessation of activity at a definite acidity is not a singular phenomenon. It is known that certain other bacteria, for example, the colon bacillus and *B. typhosus* and the allied *B. paratyphosus A* and *B*, cease growing at a fairly definite hydrogen ion concentration.

Clark and Lubs [1915, 1] found a certain correspondence in the final reaction and the type of colon bacillus with which they were working. Cole and Onslow [1916] claim to be able to differentiate the organisms of the typhosus group by the velocity with which they reach a certain acid reaction.

Previous to these observers, Brünn [1913] had shown that *B. coli* dies within twenty-four hours if exposed to an acid reaction of $P_H = 4{\cdot}7$ but not of $P_H = 5{\cdot}0$. Michaelis [1914] grew *B. coli* in lactose bouillon and found that the acidity always rose to but did not exceed $P_H = 5{\cdot}0$; but the amount of literature bearing on the subject of the effect of reaction on the growth of micro-organisms is not extensive. Very recently Clark and Lubs [1917] have

published a series of very interesting papers in which will be found many references to this important problem and which should be consulted by those interested in its history.

Attempts have been made by many investigators to estimate the effect of acid solutions on bacteria by simply adding definite amounts of various acids to media containing bacteria, or by treating the media with acids before inoculation. So far this method has yielded very little useful information, as the experimenters have for the most part neglected to take into account the influence of a complex medium—containing many buffer substances—on the dissociation of the acids. Any investigation of the effect of acids on bacteria must be preceded by a study of the effects of the acids on the media which are being used.

So far as we are aware the only attempt to determine the effect of acids on culture media in altering their hydrogen ion concentration, where modern methods have been used, is that of Clark and Lubs [1915, 2]. Their conclusions regarding the futility of titrating media, as is done in most bacteriological laboratories, are of great importance and are fully borne out by the present work. The point at which their investigation touches our own is where they show that the effect of acids on media is not directly proportional to the quantity of acid added. In this connection it should be borne in mind that different acids affect media to different extents, depending on many factors. Amongst these one may mention the dilution, the degree of dissociation of the acids and the salts contained in the medium, and the combinations they may form with the complex substances of which bacteriological media are composed.

Clark and Lubs investigated the effect of adding lactic and hydrochloric acid to milk, peptone water, etc., and expressed the results by means of titration curves, in which the amounts of acid added were plotted against the absolute reactions resulting, as measured with the hydrogen electrode. These curves show clearly the buffer action of the media; how they absorb part of the hydrogen ions of the acid and cause the reaction to be far less acid than that which the same amount of acid would produce if added to a medium free from buffer substances. Thus, milk to which sufficient acid had been added to make the solution 0·023 *N* had a reaction of $P_H = 5{\cdot}4$, which is the reaction corresponding roughly to that of a solution of hydrochloric acid only 0·000004 *N*.

Recently Taylor [1917] has been working on the effect of acids on the growth of bacteria and claims to have obtained certain specific effects with

certain acids. He has, however, made no attempt to show what effect is due to the anions of the acid and what is due to that cation which is common to them all—namely the hydrogen ion.

This matter is of importance, for in the work of Wolf and Telfer [1917] a study has been made of the acids involved in the fermentation of *B. perfringens* and *B. sporogenes*. Should any of the acids which are normally found as the fermentation products of these organisms have a specific effect we should be inclined to their use in combating local infection. This specific action of acids cannot be excluded without experimental evidence to the contrary, for we know that a large number of anions have a very decided inhibitory effect on the growth of bacteria.

In the following experiments it will be shown that *B. perfringens* fails to grow in a glucose peptone medium containing sufficient hydrochloric acid to give the solution a reaction of $P_H = 4{\cdot}8$. This reaction corresponds to that of a solution of hydrochloric acid only 0·000016 *N* and is produced by adding to the medium containing buffer substances sufficient HCl to make it 0·02 *N* with regard to the acid itself. That this inhibitory effect is not due to the anion is proved by the fact that a 0·1 *N* solution of NaCl has no toxic action on bacteria, and may even have an accelerating effect on their growth.

On the other hand, acids like benzoic, salicylic or boric, distinctly inhibit bacterial growth, and this takes place whether they are present as free acids or as sodium salts.

We have therefore examined the effects of the more commonly occurring acids to see if any of them possess antiseptic properties outside of those conferred on them by their hydrogen ion.

The four main points to which attention has been directed in the present paper are the following:

1. What is the effect of equivalent amounts of acids of differing dissociation constants on the reaction of a standard medium?

2. Knowing this effect, what is the minimum amount of acid, and the corresponding hydrogen ion concentration, which exerts a lethal effect on the organisms chosen?

3. Is there in any of these acids a discoverable effect which cannot be set down to the hydrogen ion concentration?

4. Is the final hydrogen ion concentration, after the cessation of growth of the organisms, a so-called "physiological constant," or is it influenced by the initial reaction of the medium?

The question of the initial reaction of the medium is one of great interest

and may have an important bearing on the latent period of growth of these organisms. In a recent paper we showed that the latent period appears to have a connection with the amino-acid content of the medium, but there were many unexplained features, which we now think may have been due largely to the initial reaction of the medium. We found that the period of optimum growth on milk coincided with a very definite hydrogen ion concentration in the case of *B. perfringens* and suggested tentatively that this might be the most favourable reaction for growth. As the result of the present experiments we have been compelled to modify our views on this point.

If growth of *B. perfringens* and *B. sporogenes* ceases at a definite reaction, it is important to determine it, for in this knowledge we may have an additional weapon for the local treatment of infections of this type. As the hydrogen ion concentrations with which we are dealing are of the order of 1/50,000 *N*, it is obvious that one cannot employ highly dissociated acids of this strength, because the least trace of protein, amino-acid, or alkali from the walls of the containing vessel would at once transform this acid solution to a neutral one. It is necessary therefore to submit the organisms to these concentrations of hydrogen ions in media containing large concentrations of buffer substances, where the action of the glass or of the carbon dioxide of the air will have a much smaller or even negligible effect.

Experimental Methods.

Preparation of Media. Titration Curves.

When an acid is added to a medium such as glucose peptone water, the resulting reaction depends on several factors. Firstly, there is the buffer action of the solution itself, due to the presence of amino-acids, phosphates, etc. The degree of dissociation of the acid has also to be considered and that of the salts it may form. As a preliminary measure, therefore, we investigated the changes of reaction of the medium to which known amounts of various acids had been added.

The medium employed was a solution of peptone made by the tryptic digestion of caseinogen. This medium is quite definite in its properties and work that we have done has shown it to be very suitable for the development of *B. perfringens* and *B. sporogenes*, with or without the addition of glucose. As the presence of glucose causes a more vigorous fermentation with each organism, the solutions used in these experiments were prepared so as to contain 2 % of this sugar.

The effect of the following acids was tried: hydrochloric, lactic, formic, butyric, acetic, malic, and succinic[1].

For each acid a titration curve was drawn (Fig. 1), showing the relationship between the amount of acid contained in the medium and the absolute reaction resulting from its presence.

The following procedure—that for hydrochloric acid—was followed for each acid used, with slight modifications as to the amounts added. A double strength solution of glucose peptone water was prepared, containing 4 % of glucose and 4 % of peptone. Eight portions of 25 cc. of this were taken and pipetted into 50 cc. graduated measuring flasks, and the amounts of $N/10$ acid and water shown in Table I were added.

This gave eight lots of 2 % glucose peptone water, differing only in the amount of acid contained per 50 cc. Each lot was divided amongst five tubes containing Durham tubes, plugged and sterilised at 110° for forty-five minutes. The tubes were of hard glass and were specially selected so as to be uniform. Their dimensions were 1·5 cm. × 16·0 cm.

After sterilisation, the reaction of each lot of tubed medium was determined at 20° electrometrically, using the hydrogen electrode, the value of P_H being calculated from the E.M.F. obtained experimentally.

In later experiments only one tube from each lot was used for determining the reaction, as it was found that there was little or no difference between corresponding tubes. The remaining tubes were used for the fermentation experiments described later.

When the reactions had been measured they were plotted against the amount of acid added. The amount of acid was recorded as cc. of $N/10$ acid contained in 10 cc. of the 2 % broth. The results for hydrochloric acid and for the other acids employed are given in Table I and Fig. 1. It will be seen from the curves that the amounts of different acids required to produce the same reaction vary considerably. Highly dissociated acids, such as hydrochloric and lactic, produce a given reaction when added in much smaller quantities than weaker acids such as acetic and butyric. These latter acids, too, soon arrive at a value of P_H where large additions of acid cause only slight changes in the value of P_H; that is to say, they are very highly influenced by the action of the buffer, the peptone solution.

[1] An attempt was made to obtain a titration curve for trichloroacetic acid, and also to observe the effect of this acid on the growth of organisms. As might have been foreseen, however, the acid decomposed during the sterilisation, yielding chloroform and carbon dioxide. These latter passed off as vapour and the media remaining possessed the reaction of ordinary glucose peptone water to which no acid had been added. On inoculation with *B. perfringens* the whole of the tubes fermented vigorously.

TABLE I. *Data for Titration Curves.*

Hydrochloric Acid.

Tube No.	50 cc. of broth made up from 25 cc. double strength broth and: cc. of *N*/10 acid	water cc.	Amount of *N*/10 acid contained in 10 cc. of broth cc.	Reaction, P_H
1	0	25·0	0	6·63
2	3·0	22·0	0·6	6·03
3	4·5	20·5	0·9	5·78
4	6·0	19·0	1·2	5·52
5	7·5	17·5	1·5	5·30
6	9·0	16·0	1·8	4·99
7	10·5	14·5	2·1	4·75
8	12·0	13·0	2·4	4·60

Lactic Acid.

1	0	25·0	0	6·63
2	2·05	22·95	0·41	6·20
3	4·10	20·95	0·82	5·78
4	6·15	18·85	1·23	5·46
5	8·20	16·80	1·64	5·05
6	10·30	14·70	2·06	4·70
7	12·35	12·65	2·47	4·56
8	14·40	10·60	2·88	4·38
9	16·45	8·55	3·29	4·28

Formic Acid.

1	0	25·0	0	6·44
2	2·0	23·0	0·4	6·10
3	4·0	21·0	0·8	5·74
4	6·0	19·0	1·2	5·42
5	8·0	17·0	1·6	5·06
6	10·0	15·0	2·0	4·77
7	12·0	13·0	2·4	4·60
8	14·0	11·0	2·8	4·44
9	16·0	9·0	3·2	4·30

Malic Acid.

1	0	25·0	0	6·52
2	2·5	22·5	0·5	6·03
3	5·0	20·0	1·0	5·65
4	7·5	17·5	1·5	5·34
5	10·0	15·0	2·0	5·10
6	12·5	12·5	2·5	4·89
7	15·0	10·0	3·0	4·71
8	17·5	7·5	3·5	4·61

TABLE I. *Data for Titration Curves.—Continued.*

Acetic Acid.

Tube No.	50 c.c. of broth made up from 25 c.c. double strength broth and: cc. of $N/10$ acid	water c.c.	Amount of $N/10$ acid contained in 10 cc. of broth cc.	Reaction P_H
1	0	25·0	0	6·52
2	3·0	22·0	0·6	5·98
3	6·0	19·0	1·2	5·56
4	9·0	16·0	1·8	5·21
5	12·0	13·0	2·4	4·95
6	15·0	10·0	3·0	4·81
7	17·5	7·5	3·5	4·75
8	20·0	5·0	4·0	4·65
9	22·5	2·5	4·5	4·60

Butyric Acid.

1	0	25·0	0	6·63
2	2·5	22·5	0·5	6·20
3	5·0	20·0	1·0	5·80
4	7·5	17·5	1·5	5·47
5	10·0	15·0	2·0	5·26
6	12·5	12·5	2·5	5·02
7	15·0	10·0	3·0	4·90
8	17·5	7·5	3·5	4·80
9	20·0	5·0	4·0	4·76

Succinic Acid.

1	0	25·0	0	6·19
2	2·5	22·5	0·5	5·84
3	5·0	20·0	1·0	5·55
4	7·5	17·5	1·5	5·36
5	10·0	15·0	2·0	5·17
6	12·5	12·5	2·5	5·06
7	15·0	10·0	3·0	4·93
8	17·5	7·5	3·5	4·83

Reaction Series. Inoculation and Fermentation.

Following the methods outlined above, various lots of media at different reactions were prepared, using the above-mentioned acids. After sterilisation, one tube from each lot of medium was opened and its reaction determined. After a few preliminary experiments we were usually able to obtain media for each acid with reactions fairly evenly distributed between $P_H = 4·0$ and $P_H = 7·0$.

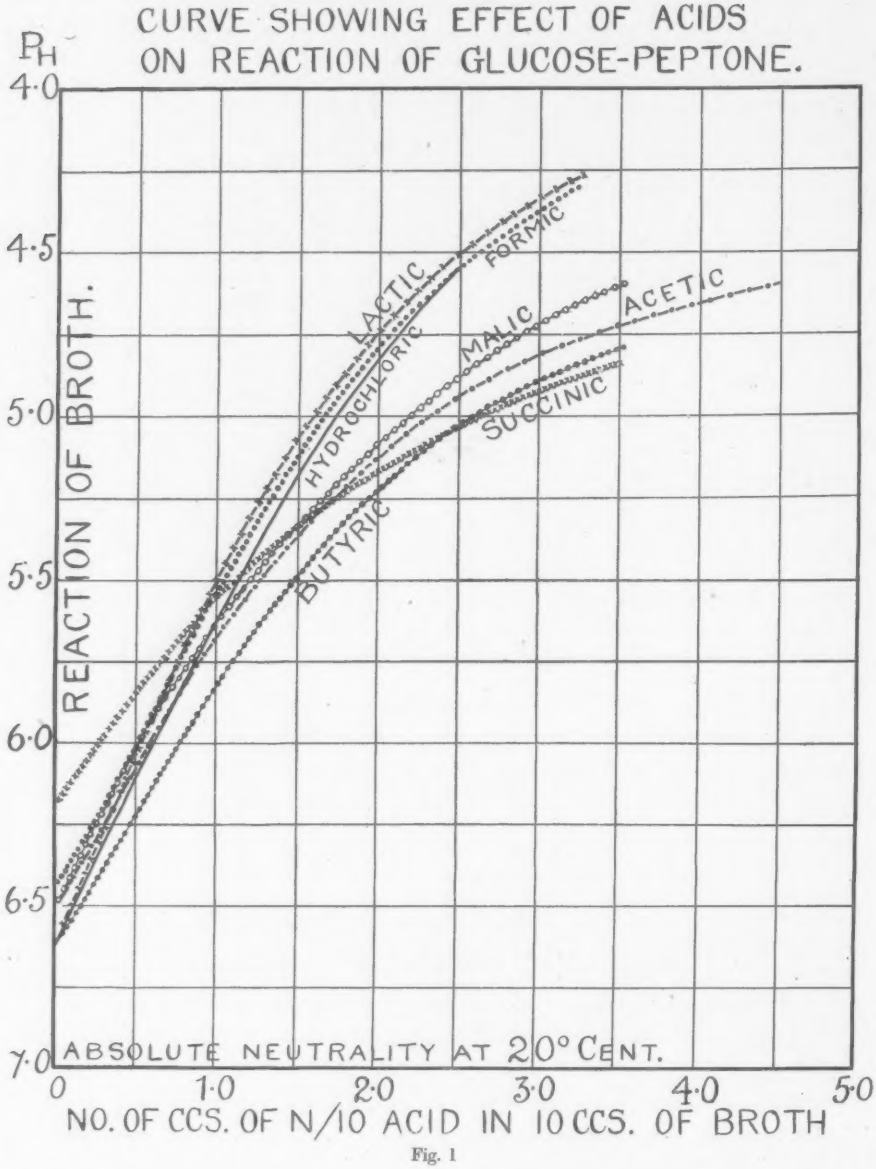

CURVE SHOWING EFFECT OF ACIDS
ON REACTION OF GLUCOSE-PEPTONE.
PH
4·0
4·5
5·0
5·5
6·0
6·5
7·0
REACTION OF BROTH.
LACTIC
FORMIC
HYDROCHLORIC
MALIC
ACETIC
SUCCINIC
BUTYRIC
ABSOLUTE NEUTRALITY AT 20° CENT.
0
1·0
2·0
3·0
4·0
5·0
NO. OF CCS. OF N/10 ACID IN 10 CCS. OF BROTH

Fig. 1

The remaining tubes were arranged into four similar "series," these latter consisting of one tube from each lot of medium. The series were called Series A, Series B, etc. The least acid member of the series was numbered 1, the others being numbered consecutively with increase of acidity.

The inoculation was made with a Pasteur pipette, each tube receiving three drops of the seed culture. As far as possible, a different culture was used for each series, so that representative results could be obtained. We were careful to use cultures, which, in our opinion, based on previous work, were vigorous.

After inoculation, the contents of each tube were covered aseptically with a layer of liquid paraffin previously sterilised, about 0·5 cm. in depth, to ensure anaerobic conditions. The necessity of this we have since shown to be open to serious doubt, but as our first experiments were conducted with the use of paraffin we continued to use it.

The inoculated tubes were now incubated at 37°. *Perfringens* cultures were allowed to remain in the incubator for three days before examination. It is advisable to give *B. sporogenes* four days to complete fermentation.

At the end of the incubation period, the series were examined and those tubes which had fermented were noted. Samples were then taken from each tube and the reaction of the fermented liquid determined, using the hydrogen electrode.

Reaction Resultants.

These final reactions proved to be of great interest and it will be well at this point to describe our method of plotting them and obtaining what we have named *reaction resultants*.

Fig. 2 shows the reaction resultant for *B. perfringens* grown on glucose peptone water acidified with varying amounts of hydrochloric acid.

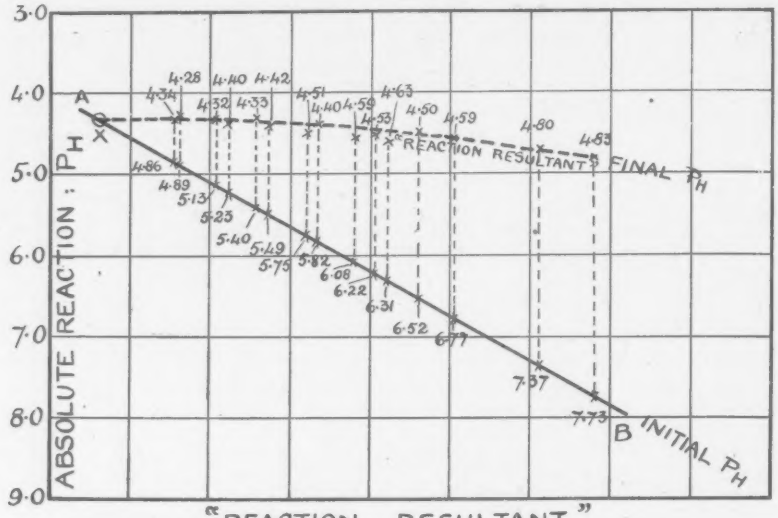
On ordinary squared paper a vertical line is taken for one axis and a scale of ordinates is chosen to represent the range of reactions over which we are working. Then at an angle of 120° to the vertical a line *AB* is drawn. This becomes the second axis. The only scale actually employed in plotting is the vertical one, as it will be seen from simple geometrical considerations that *AB* is conveniently divided into a scale by the parallel horizontal lines of the squared paper passing through the ordinates. On *AB*, the point corresponding to the initial reaction of the particular medium is marked off. Then, on a line through this point and parallel to the vertical axis, the point corresponding to the final reaction of the medium is marked. For each initial reaction marked on the line *AB*, a point for the final reaction is found in a similar manner, and

through the series of points thus obtained, a smoothed curve is drawn. This curve we have named the *reaction resultant*.

In employing this method of plotting results, what we have actually done is to make use of a pair of axes at an angle of 120° to each other, instead of being at right angles as axes usually are. The choice of the angle between the axes is only governed by considerations of general convenience. Where comparative results are required, the angle must necessarily be definitely fixed.

In Fig. 2, the reaction resultant is derived from large numbers of fermentations in which *B. perfringens* has acted on glucose peptone water containing various added amounts of hydrochloric acid. In the figure, only fifteen points are shown owing to lack of space, but we have many other confirmatory points from additional experiments.

It will be observed that the points recording the final values of P_H lie very close to the smooth curve that has been drawn to represent them, and establish quite definitely the form of this line.



"REACTION RESULTANT."
B. PERFRINGENS ON GLUCOSE-PEPTONE.
ACID REACTIONS PRODUCED BY HYDROCHLORIC ACID.

Fig. 2

Bacillus perfringens.

Data are given below for experiments carried out with *B. prefringens* on glucose peptone water in the presence of various acids.

The tables show the initial and final reactions of the media in which fermentation took place. In cases where the bacillus was able to ferment the medium a positive sign (+) is placed under the heading "Result"; where no fermentation took place there is a negative sign (−).

It was found that in tubes where there was no sign of fermentation—as evidenced by the absence of gas or turbidity in the medium—the reaction remained unchanged at the end of the incubation period.

The final reactions were plotted against the initial reactions by the method previously described. When two or three similar series of tubes were used, the mean value of the final P_H for each lot of medium was taken for the purpose of plotting the reaction resultant.

Special interest is attached to the particular value of initial P_H at which the bacillus loses its power to ferment, and this has been noted in the following experimental data.

It should be noted that the reactions of the media were determined at 20°, whereas the fermentations took place in the incubator at 37°. At the higher temperature, the media would have a higher acidity than at 20°. The difference is not considerable but cannot be overlooked.

We have not made experiments touching the change in reaction of the media with change in temperature, but if we may be allowed to use Michaelis' figures for sodium acetate—acetic acid mixtures which will probably serve the purpose, 0·8 millivolt may be subtracted for each degree above 20°.

This would give, for a rise of 17°, a decrease in E.M.F. of 13·6 mv. This correction on a P_H of 4·67 would give a final corrected value of $P_H = 4·45$.

Experiment No. 1 (Laboratory Experiment R. S. 1). Hydrochloric acid and sodium hydroxide.

In this preliminary experiment not only was hydrochloric acid added to the peptone water, but also sodium hydroxide to some of it in order to give media with a reaction above $P_H = 7·0$. There was every sign of caramelisation of these alkaline media during the sterilisation, and the experiments were discontinued[1].

Data (see also Fig. 3).

Series A. Inoculated with K 71 (thirteen hours old on milk).

[1] The effect of sterilisation on the reaction of media was investigated by the older methods by Hesse [1904]. He observed an increase in titratable acidity after heating. Clark and Lubs [1917] have investigated the change using the hydrogen electrode and have found an increase in P_H ranging from 0·01 with Witte's peptone to 0·44 with skimmed milk. The change is partly dependent on the carbohydrate content of the medium and the hydrogen ion concentration of the solution. By oxidation organic acids are formed. The caramelisation is due to melanines, formed by interaction of amino-acids and glucose [Maillard, 1913].

Series B. Inoculated with K 72 (thirteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 3, Series A, with value of $P_H = 4{\cdot}89$.

The next set of tubes, with $P_H = 4{\cdot}3$, failed to ferment.

TABLE II. (*Exp.* 1.)

No. of tube	Initial reaction, P_H	Series A		Series B	
		Result	Final reaction, P_H	Result	Final reaction, P_H
1	3·9	–	3·87	–	3·92
2	4·3	–	4·33	–	4·33
3	4·89	+	4·28	–	4·88
4	5·78	+	4·40	+	4·38
5	6·77	+	4·59	+	4·59
6	7·37	+	4·75	+	4·83
7	7·73	+	4·83	+	4·80
8	8·1	+	4·80	+	4·76

Experiment No. 2 (Laboratory Experiment R. S. 2). Hydrochloric acid.

Data (see also Fig. 3).

Series A. Inoculated with K 80 (fifteen hours old on milk).

Series B. Inoculated with K 78 (fifteen hours old on milk).

Series C. Inoculated with K 79 (fifteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 5, Series A, with value of $P_H = 4{\cdot}86$.

The next set of tubes, with $P_H = 4{\cdot}60$, failed to ferment.

TABLE III. (*Exp.* 2.)

No. of tube	Initial reaction, P_H	Series A		Series B		Series C	
		Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·22	+	4·54	+	4·52	+	4·53
2	5·82	+	4·40	+	4·44	+	4·37
3	5·40	+	4·33	+	4·33	+	4·35
4	5·13	+	4·34	+	4·32	–	...
5	4·86	+	4·34	–	...	–	...
6	4·60	–	...	–	...	–	...
7	4·36	–	...	–	...	–	...
8	4·19	–	...	–	...	–	...
9	3·78	–	...	–	...	–	...

Experiment No. 3 (Laboratory Experiment R. S. 5). Lactic acid.

Data (see also Fig. 3).

Series A. Inoculated with K 107 (twelve hours old on milk).

Series B. Inoculated with K 109 (twelve hours old on milk).

Series C. Inoculated with K 108 (twelve hours old on milk).

TABLE IV. (*Exp.* 3.)

No. of tube	Initial reaction, P_H	Series A Result	Series A Final reaction, P_H	Series B Result	Series B Final reaction, P_H	Series C Result	Series C Final reaction, P_H
1	6·19	+	4·60	+	4·57	+	4·58
2	5·46	+	4·49	+	4·51	+	4·49
3	5·15	+	4·51	+	4·51	+	4·51
4	4·85	+	4·80	−	...	−	...
5	4·61	−	...	−	...	−	...
6	4·42	−	...	−	...	−	...
7	4·26	−	...	−	...	−	...

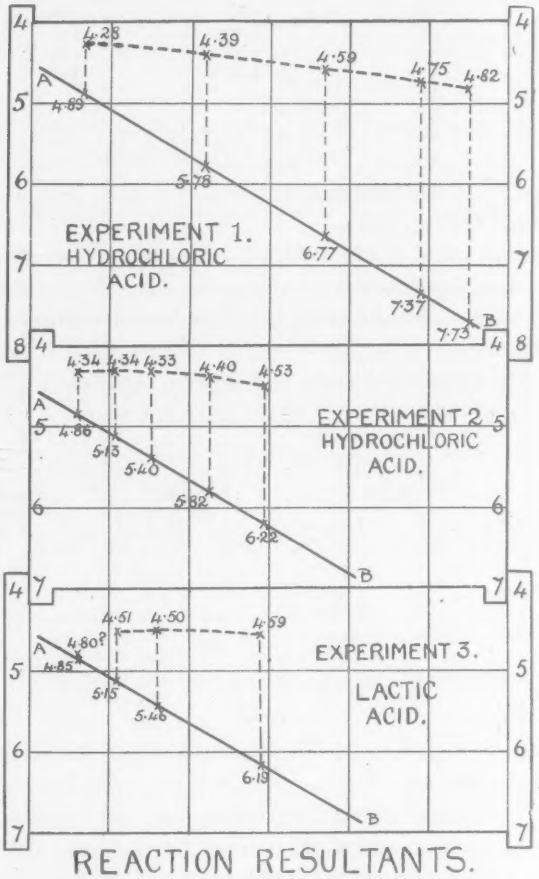


Fig. 3

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 4, Series A, with value of $P_H = 4{\cdot}85$. The fermentation was only very slight.

The next set of tubes, with $P_H = 4{\cdot}61$, failed to ferment.

TABLE V. (*Exp.* 4.)

No. of tube	Initial reaction, P_H	Series A		Series B	
		Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·44	+	4·50	+	4·50
2	6·10	+	4·39	+	4·39
3	5·74	+	4·39	+	4·41
4	5·42	+	4·40	+	4·44
5	5·06	+	4·60	?	4·81
6	4·77	−	...	−	...
7	4·60	−	...	−	...
8	4·44	−	...	−	...
9	4·30	−	...	−	...

Experiment No. 4 (Laboratory Experiment R. S. 12). Formic acid.

Data (see also Fig. 4).

Series A. Inoculated with K 127 (twenty-four hours old on cooked meat).

Series B. Inoculated with K 123 (ninety-six hours old on cooked meat).

The tubes were examined seventy-two hours after inoculation.

The most acid tubes to ferment were Nos. 5, Series A and B, with value of $P_H = 5{\cdot}06$. The fermentations were very slight in each case.

The next set of tubes, with $P_H = 4{\cdot}77$, failed to ferment.

TABLE VI. (*Exp.* 5.)

No. of tube	Initial reaction, P_H	Series A		Series B		Series C	
		Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·52	+	4·52	+	4·51	+	4·49
2	6·03	+	4·46	+	4·46	+	4·45
3	5·65	+	4·38	+	4·38	+	4·39
4	5·34	+	4·38	+	4·38	+	4·39
5	5·10	+	4·37	+	4·39	+	4·37
6	4·89	−	...	+	4·37	+	4·38
7	4·71	−	...	+	4·38	+	4·36
8	4·60	−	...	−	...	−	...

Experiment No. 5 (Laboratory Experiment R. S. 8). Malic acid.

Data (see also Fig. 4).

Series A. Inoculated with K 118 (thirteen hours old on milk).

Series B. Inoculated with K 119 (thirteen hours old on milk).

Series C. Inoculated with K 119 (thirteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tubes to ferment were Nos. 7, Series B and C, with value of $P_H = 4{\cdot}71$.

The next set of tubes, with $P_H = 4{\cdot}60$, failed to ferment.

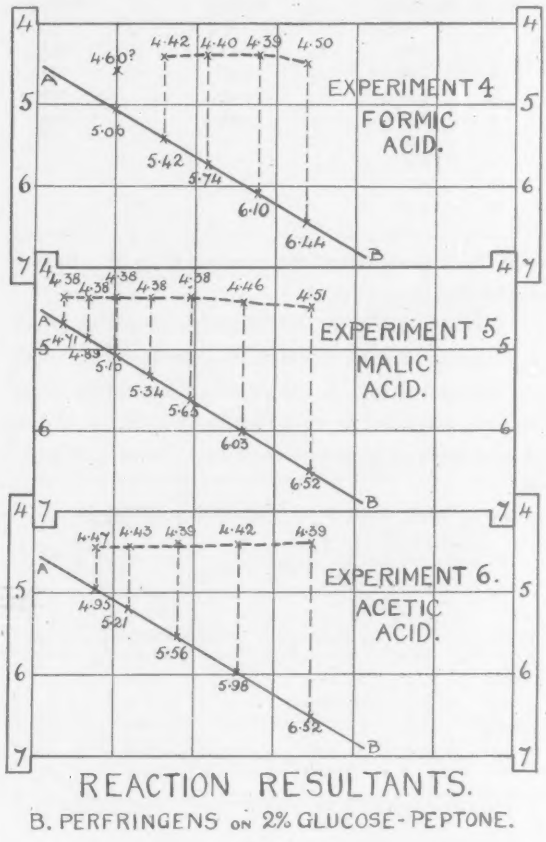


Fig. 4

Experiment No. 6 (Laboratory Experiment R. S. 13). Acetic acid.

Data (see also Fig. 4).

Series A. Inoculated with K 120 (twenty-four hours old on cooked meat).

Series B. Inoculated with K 121 (twenty-four hours old on cooked meat).

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 5, Series B, with value of $P_H = 4{\cdot}95$. The next set of tubes, with $P_H = 4{\cdot}81$, failed to ferment.

TABLE VII. (*Exp.* 6.)

		Series A		Series B	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·52	+	4·38	+	4·41
2	5·98	+	4·43	+	4·41
3	5·56	+	4·39	+	4·39
4	5·21	+	4·43	+	4·44
5	4·95	–	...	+	4·47
6	4·81	–	...	–	...
7	4·75	–	...	–	...
8	4·65	–	...	–	...
9	4·60	–	...	–	...

Experiment No. 7 (Laboratory Experiment R. S. 4). Butyric acid.

Data (see also Fig. 5).

Series A. Inoculated with K 102 (fifteen hours old on milk).

Series B. Inoculated with K 103 (fifteen hours old on milk).

Series C. Inoculated with K 101 (fifteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tubes to ferment were Nos. 5, Series A, B and C, with value of $P_H = 5{\cdot}03$.

The next set of tubes, with $P_H = 4{\cdot}96$, failed to ferment.

TABLE VIII. (*Exp.* 7.)

		Series A		Series B		Series C	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·33	+	4·52	+	4·50	+	4·52
2	5·62	+	4·48	+	4·48	+	4·48
3	5·39	+	4·64	+	4·60	+	4·60
4	5·17	+	4·61	+	4·64	+	4·57
5	5·03	+	4·67	+	4·67	+	4·65
6	4·96	–	...	–	...	–	...
7	4·83	–	...	–	...	–	...
8	4·80	–	...	–	...	–	...
9	4·68	–	...	–	...	–	...

Experiment No. 8 (Laboratory Experiment R. S. 6). Succinic acid.

Data (see also Fig. 5).

Series A. Inoculated with K 113 (fourteen hours old on milk).

Series B. Inoculated with K 114 (fourteen hours old on milk).

Series C. Inoculated with K 115 (fourteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

TABLE IX. (*Exp.* 8.)

No. of tube	Initial reaction, P_H	Series A		Series B		Series C	
		Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·19	+	4·52	+	4·52	+	4·51
2	5·84	+	4·50	+	4·49	+	4·48
3	5·55	+	4·51	+	4·52	+	4·52
4	5·36	+	4·54	+	4·54	+	4·53
5	5·17	+	4·56	+	4·56	+	4·56
6	5·06	+	4·58	+	4·57	+	4·57
7	4·93	+	4·57	+	4·56	−	...
8	4·83	−	...	−	...	−	...

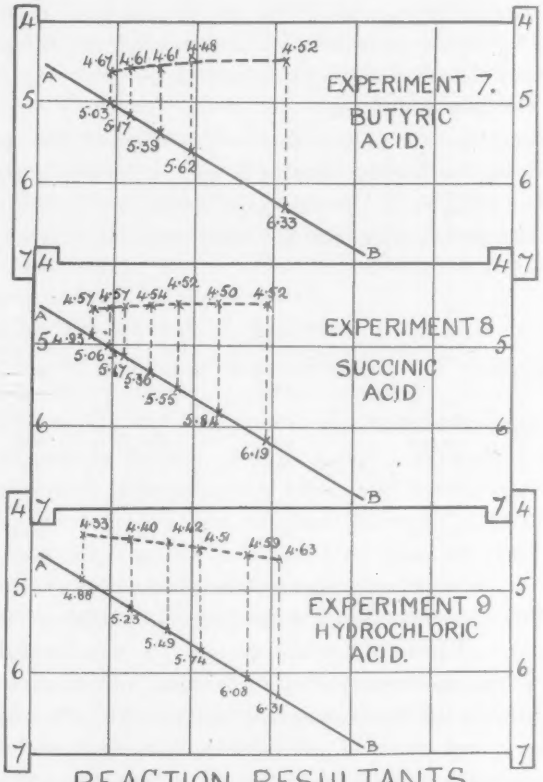


Fig. 5

The most acid tubes to ferment were Nos. 7, Series A and B, with value of $P_H = 4{\cdot}93$.

The next set of tubes, with $P_H = 4{\cdot}83$, failed to ferment.

One of the first things noticed in these experiments was that increasing the initial acidity of a medium had an effect on the latent period. Thus, when a series of tubes was inoculated with the same seed at the same time, the least acid member of the series was the first to ferment. The more acid the reaction of the medium the longer the latent period, showing that whatever may be the effect of the acidity on the subsequent fermentation it has a large influence in determining the latent period.

With *B. perfringens*, a glucose peptone solution begins to ferment in from four to five hours after inoculation when the reaction of the medium is from $P_H = 7{\cdot}0$ to $P_H = 6{\cdot}2$. As more acid media are employed, the latent period lengthens, until, for a medium with an initial reaction of 4·9, the latent period may last about forty-eight hours.

In reviewing the data given above it will be observed that the values of P_H at which the bacillus just manages to grow all lie close together in the neighbourhood of $P_H = 4{\cdot}8$. In the following table are given the most acid values at which growth takes place and those which just prevent it.

TABLE X.

Experiment No.	Acid	Most acid value of P_H which allows growth	Least acid value of P_H which inhibits growth	Mean value of P_H. Critical P_H
2	Hydrochloric	4·86	4·60	4·78
3	Lactic	4·85	4·61	4·68
4	Formic	5·06	4·77	4·91
5	Malic	4·71	4·60	4·66
6	Acetic	4·95	4·81	4·88
7	Butyric	5·03	4·96	4·99
8	Succinic	4·93	4·83	4·88

Mean value of critical $P_H = 4{\cdot}82$

With a large number of experiments it might be possible to find the exact value of P_H for each acid which would just permit growth, the slightest excess in acidity above this value preventing growth. In our experiments the differences between the "permitting reaction" and the "inhibiting reaction" are not great in any case, and probably the mean of the two values found experimentally is not far from this *critical* P_H. These mean values are given in the fifth column of the above table, and it will be observed that they are quite close together.

The following table shows the amount of each acid that must be present in the glucose peptone water to give these critical reactions. The amounts are determined from the titration curves (Fig. 1).

TABLE XI.

Acid	Critical P_H	cc. of $N/10$ acid contained in 10 cc. of broth	Acidity of medium in terms of N acid	Molar strength of medium in acid	G. of acid per litre of medium
Hydrochloric	4·78	2·05	0·0205 N	0·0205 M	0·748
Lactic	4·68	2·10	0·0210 N	0·0210 M	1·910
Formic	4·91	1·80	0·0180 N	0·0180 M	0·828
Malic	4·66	3·50	0·0350 N	0·0175 M	2·335
Acetic	4·88	2·70	0·0270 N	0·0270 M	1·620
Butyric	4·99	2·60	0·0260 N	0·0260 M	2·288
Succinic	4·88	3·20	0·0320 N	0·0160 M	1·888

The titration curves in Fig. 1 show the comparative strengths of the acids in presence of glucose peptone water. Thus, lactic, hydrochloric and formic acids behave as strong acids compared with acetic, butyric and succinic acids. It will be seen from the above table that the strong acids are more effective in inhibiting growth than the weaker acids when present in equivalent concentrations. Thus, when hydrochloric is present in sufficient quantity to make the medium 0·0205 N the critical reaction for *B. perfringens* is reached. On the other hand enough succinic acid must be present to give a 0·032 N solution before the critical reaction is attained.

These facts lead one to think that the dominant factor in the development of *B. perfringens* is not so much the amount of acid present as the *acidity* that the acid produces.

It seems that at about $P_H = 4·8$ there is a point where the reaction just permits the organism to develop. A slight excess in hydrogen ion concentration over this value prevents growth. Each acid may have some slight effect of its own, due to its molecular structure. Thus, malic acid is to some extent an anomaly, allowing growth to take place when the value of P_H is 4·71. Butyric acid on the other hand has a high value for its critical P_H (4·99), and seems to have a slight inhibiting effect in addition to that produced by its hydrogen ions.

On the whole it appears that the reaction due to the acid is more important than the actual amount or molecular quality of the acid in determining the growth or non-development of *B. perfringens*.

We now come to a consideration of the final reactions of the media after fermentation by *B. perfringens*. These final reactions are given in the tables and represented graphically in the reaction resultants.

These results show that the final reaction at which growth ceases is by no means a "physiological constant." On the contrary, it varies not only with the reaction of the medium before inoculation but also with the acid used to produce this particular reaction.

With hydrochloric acid (Fig. 2), when the initial reaction is $P_H = 6{\cdot}77$, growth stops at the point where the bacillus has produced sufficient acid to give the medium a reaction of $P_H = 4{\cdot}59$. For an initial reaction of $P_H = 4{\cdot}86$, however, the final reaction is $P_H = 4{\cdot}34$. For initial reactions between the two, final reactions are obtained which take up orderly positions between $P_H = 4{\cdot}59$ and $P_H = 4{\cdot}34$, and give the curve shown in the figure. There is therefore a very definite relation between the initial and final reactions.

Recently, in connection with *B. coli*, attempts at diagnosis have been made in which the final reaction of a particular medium when inoculated with the organism was determined. In view of the above results it would seem that great care must be exercised in using such a method. In order to use the final reaction of a fermentation as a criterion for a given organism, absolute uniformity of the medium is essential. This, applies especially to the hydrogen ion concentration, on which, as will be seen, the final reaction depends.

In examining the curves for the other acids used, one finds that the question of the final reaction becomes further complicated. Here, for the first time in these experiments, one sees signs of specificity in the action of the acids. The reaction resultants for hydrochloric, lactic and malic acids show that the final reactions of the media become definitely more acid as the initial hydrogen ion concentration increases. On the other hand, with butyric, succinic and acetic acids, the reverse rule holds good. The experiments appear to bring out the important point that all acids, in the presence of which *B. perfringens* can develop, probably give characteristic reaction resultants, as do the seven acids with which we have worked. Considerable interest is attached to the point *X* (Fig. 2), obtained by the intersection of the reaction resultant produced and the line *AB*. The reaction corresponding to this point might be considered theoretically as that which definitely stops the growth of *B. perfringens*. It should be remarked, however, that we have never been able to cause a medium having this reaction to ferment, no doubt on account of inhibiting influences other than the reaction. The value obtained at the point *X* differs for each individual acid. This, in itself, is an indication of specific action.

It is necessary to search closely for the factors responsible for these resultants. When *B. perfringens* ferments glucose peptone water, it forms,

as Wolf and Telfer [1917] have shown, a mixture of lactic acid, butyric acid and small amounts of other low members of the fatty acid series.

How far the *quality* of these acids is affected by the nature of the acid added to obtain the required initial reaction one is unable to say at present. The reaction resultants, however, will give a fair idea of the *quantity* of acids produced, provided one has some notion of the factors which influence them. To this end it will be necessary to take a specific example.

Let it be assumed that a series of flasks of glucose peptone water are prepared differing from one another in their initial hydrogen ion concentration, this effect being produced by the addition of increasing amounts of butyric acid to the medium. These flasks are then inoculated with an organism producing butyric acid exclusively. The quantity of butyric acid produced is also assumed to be independent of the initial reaction of the medium and is a constant amount. At the end of the fermentation the reaction resultants are plotted.

We have already determined the changes in reaction of glucose peptone caused by the addition of various amounts of butyric acid. These results can be expressed graphically in a titration curve, which will aid us to construct the reaction resultants for the above hypothetical fermentation. All that it is necessary to do is to select on the titration curve a number of points and consider the reactions for these points as the initial reactions of the media. To the quantity of acid corresponding to each point a definite amount of acid is added, and the reactions corresponding to the total amounts noted. These reactions will be the final reactions for the fermentation, and when plotted in the usual way against the initial reactions will give the reaction resultant. Thus, one can construct the reaction resultants for the production of any amount of acid providing one has a suitable titration curve. This has been done for four hypothetical fermentations (Fig. 6, I).

In the first case the fermentation is supposed to produce butyric acid equivalent to 3 cc. of $N/10$ acid per 10 cc. of broth; in the second, 5 cc.; in the third, 7 cc.; in the fourth, 9 cc. It is seen that the reaction resultants have a very definite form. They rise steadily from right to left on the paper, and the four curves tend to meet at a point on AB at which an addition of butyric acid will not make the reaction more acid. Different acids will, of course, give different curves, the general form of the reaction resultant, however, will be the same, providing the amount of acid produced does not vary. They will all rise from right to left of the paper towards the value of P_H where the acidity is not increased by further addition of the acid. It is clear also

that the resultant arising from the added acid must always approach the line *AB* tangentially, when a constant amount of acid is being formed.

Now let it be imagined that the accumulation of acids hinders the bacteria and causes their production of acid to fall off steadily in proportion to the concentration of acid present. The reaction resultants will then take the form

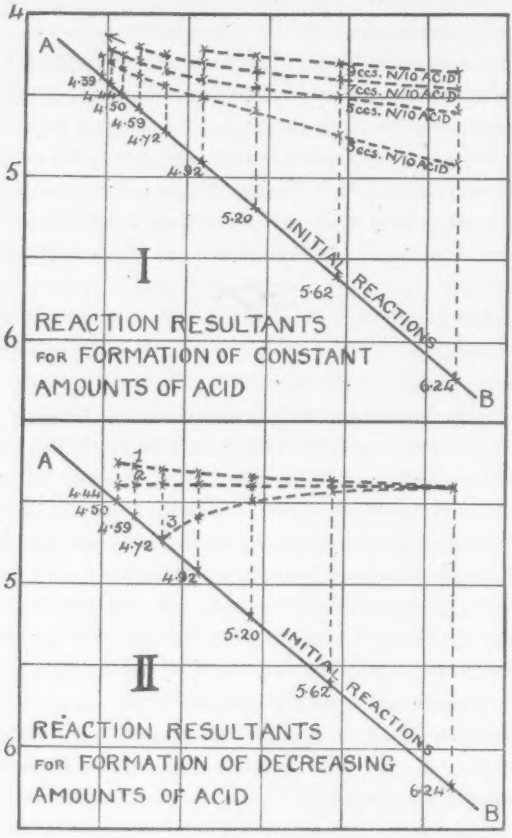


Fig. 6

shown in the second diagram (Fig. 6, II). We give the curves for three cases. In curve No. 1 the inhibiting influence is made slight, in No. 2 more marked, and in No. 3 greater still.

From a consideration of this imaginary fermentation one can now see how, from the reaction resultants, it can be estimated to what extent the acids have

affected fermentation. If the curve rises from right to left and tends to approach *AB* tangentially it means that the amount of acid produced is not falling off very greatly as the initial hydrogen ion concentration increases. If, on the other hand, the concentration of acids is causing a decrease in fermenting power, the reaction curves will tend to fall from right to left, according to the magnitude of the inhibiting influences. Where the bacteria cease activity as soon as a definite *concentration* of acid is reached, the reaction resultant will be a horizontal line (Fig. 6, II, 2).

From our experiments we have obtained various reaction resultants. The one most nearly approaching the case where the fermentation is not hindered by the accumulation of acids is with hydrochloric acid.

The following series of fermentations will show to what extent the foregoing considerations are borne out experimentally, and how far one may rely on conclusions as to acid formation and intensity of metabolism drawn from the reaction resultants.

Experiment No. 9 (Laboratory Fermentations Nos. 42–47).

Into each of six 500 cc. Kjeldahl flasks were placed 250 cc. of 2 % glucose peptone medium, as used in the foregoing reaction experiments. During preparation varying amounts of hydrochloric acid had been added to each lot of medium so that we had six broths exactly similar, except in so far as the amounts of contained acid, and consequently the reactions, were concerned. The flasks were plugged and sterilised in the ordinary way, afterwards being inoculated each with 1 cc. of the same culture of *B. perfringens*. The plugs were then pushed lower into the necks of the flasks and well fitting rubber stoppers, each provided with a short delivery tube, inserted. By means of a Fleuss pump the flasks were now evacuated by way of the delivery tubes, which were then closed off by means of a piece of pressure tubing and a screw clip. All joints were made tight with hot paraffin wax. The flasks were incubated for four days. At the end of this time the gas production was measured and a determination made of the amount of acid produced. To measure the gas production, the flasks were connected to an evacuated mercury manometer, the clip unscrewed, the pressure of the gas determined, and hence its volume calculated.

A sample of the gas was in each case analysed. From each flask also a portion of the liquid was taken and the final P_H measured electrometrically.

The amounts of volatile and fixed acids were determined by Captain Telfer, R.A.M.C., to whom our acknowledgements are due. The total volatile acids

were determined by exhaustive steam distillation and the fixed acids by ethereal extraction of the residue in a liquid extractor. As the final stages of the distillation are accompanied to some extent by volatilisation of the fixed acids, and as also the final traces of lower members of the fatty acid series are only removed with difficulty, we have, in tabulating our results, added together the amounts of volatile and fixed acids, and taken their sum to represent the total amount of acids produced.

TABLE XII. (*Exp.* 9.)

No. of Fermentation	Initial P_H	Gas production			Final P_H	Acid production cc. of $N/10$ acid per litre of broth		
		cc. per litre	CO_2 %	H_2 %		Volatile acids cc.	Fixed acids cc.	Total acids cc.
F 42	6·31	1036	42·3	57·7	4·63	232	171	403
F 43	6·08	1096	40·5	59·5	4·59	259	196	455
F 44	5·74	1000	41·6	58·4	4·51	200	213	413
F 45	5·49	888	41·3	58·7	4·42	156	231	387
F 46	5·23	916	41·0	59·0	4·40	229	205	434
F 47	4·88	940	...	...	4·33	181	241	422

It will be seen from the above table that in the final reactions alone can we trace any sign of progressive relation between the final measurements on the different media. These final reactions have been plotted against the initial reactions (see Fig. 5), and give an ordinary reaction resultant such as has been found characteristic for hydrochloric acid.

The gas analyses indicate that the products of fermentation are very similar, differing as they do to only the smallest degree. One sees too that the final amounts of gas produced are so close that they may be considered equal, as may also the total amounts of acid. What differences we have obtained in the individual experiments may fairly be considered as due to experimental errors.

These results show that the fermentation of *B. perfringens* is very little affected by the addition of hydrochloric acid, up to a quantity sufficient to give the critical reaction.

We hope to carry out similar experiments with the other acids involved, but such experiments are long and laborious and for the moment one can obtain a large amount of information merely from the reaction resultants.

Lactic and malic acids, which act as strong acids, apparently play a less passive role than hydrochloric acid. The final products, judging from the resultants, fall off somewhat as the initial acidity rises. With the weaker acids, succinic and butyric (and it should be noted that the absolute strength

of these acids in the presence of peptone water can be accurately judged from the titration curves), there is a greater inhibiting effect shown. Acetic acid, which behaves more or less as an intermediate acid from the point of view of strength, has a corresponding intermediate resultant.

Formic acid behaves slightly anomalously, apparently having a rather larger inhibiting effect than acids of similar strength. The aldehyde grouping of the acid may be a partial explanation of this fact.

Generally speaking, then, we find little specificity of action in the acids as regards the initial P_H, but a greater difference in effect towards the final reaction. To what can this be ascribed? The best explanation seems to be that the inhibiting action of these acids, as shown by slackened fermentations, is due to an effect of *mass*. It should be remembered that we have been working throughout over the same range of initial reactions; also that to produce these initial reactions a larger amount of a weak acid is necessary than of a strong acid. Therefore, if we assume that all of these acids, considered merely as foreign substances, have an effect in concentrated solutions independent of their P_H but proportional to their mass, we can see why in the case where a weak acid is used to give the initial reaction the fermentations are partially inhibited. On the other hand, a strong acid, such as hydrochloric acid, only present in small molecular quantities, has little effect.

Summing up therefore these results, it would seem that the seven acids under consideration have a definite effect on *B. perfringens* proportional to the concentration of their common cation—hydrogen ion. Their anions, on the other hand, and the undissociated acids merely behave as foreign bodies, possessing a certain inhibiting effect proportional to their molecular concentration.

At some future date we hope to show the effect of adding various amounts of neutral salts of these acids to peptone water. We can obtain a constant initial reaction by adding hydrochloric acid which we have shown to have little effect on the fermentations. By this method we shall be able to estimate accurately the effect of the anions of the acids.

Bacillus sporogenes.

Experiments similar to those described above with *B. perfringens* were carried out with *B. sporogenes*. Glucose peptone water was again used as the fermentation medium, and the effect was tried of the following acids: hydrochloric, formic, lactic, acetic and butyric.

With the *B. sporogenes* the fermentation is accompanied by the formation

of a base, ammonia, which, although large amounts of acid are produced, causes the final reaction to be less acid than those resulting from *B. perfringens*.

The experimental details were similar. Readings were taken of the final reactions and the reaction resultants plotted.

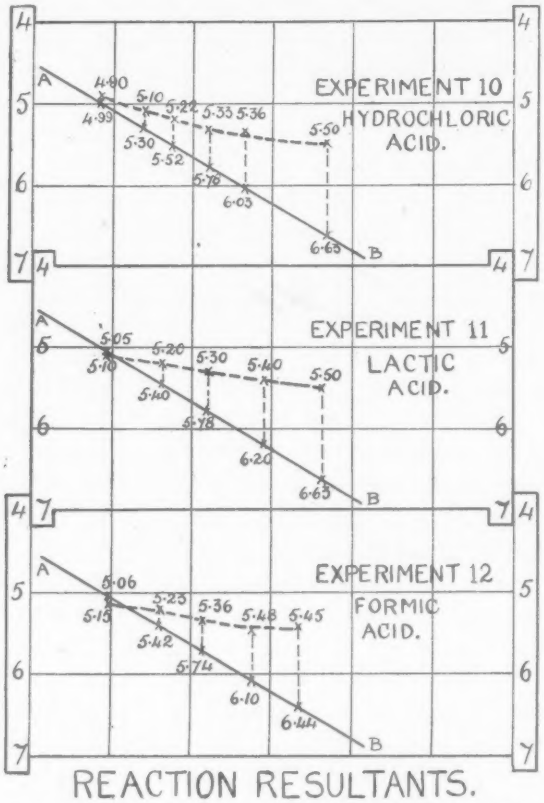


Fig. 7

B. sporogenes acts rather slower than *B. perfringens* and it was found necessary to continue the incubation for a fourth day, making ninety-six hours in all, before the final reactions were determined.

Experiment No. 10 (Laboratory Experiment R. S. 9). Hydrochloric acid.

Data (see also Fig. 7).

Series A. Inoculated with D 74 (forty-eight hours old on cooked meat).

Series B. Inoculated with D 75 (1) (forty-eight hours old on milk).

Series C. Inoculated with D 75 (2) (forty-eight hours old on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tubes to ferment were Nos. 6, Series B and C, with value of $P_H = 4{\cdot}99$.

The next set of tubes, with $P_H = 4{\cdot}75$, failed to ferment.

TABLE XIII. (*Exp.* 10.)

		Series A		Series B		Series C	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·63	+	5·51	+	5·45	+	5·52
2	6·03	+	5·39	+	5·34	+	5·35
3	5·78	+	5·37	+	5·31	+	5·31
4	5·52	+	5·22	+	5·20	+	5·23
5	5·30	+	5·07	+	5·10	+	5·08
6	4·99	−	...	+	4·91	+	4·90
7	4·75	−	...	−	...	−	...
8	4·60	−	...	−	...	−	...

Experiment No. 11 (Laboratory Experiment R. S. 11). Lactic acid.

Data (see also Fig. 7).

Series A. Inoculated with D 74 (forty-eight hours on cooked meat).

Series B. Inoculated with D 75 (1) (forty-eight hours on milk).

Series C. Inoculated with D 75 (2) (forty-eight hours on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tube to ferment was No. 5, Series C, with value of $P_H = 5{\cdot}05$.

The next set of tubes, with $P_H = 4{\cdot}70$, failed to ferment.

TABLE XIV. (*Exp.* 11.)

		Series A		Series B		Series C	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·63	+	5·51	+	5·45	+	5·52
2	6·20	+	5·49	+	5·34	+	5·37
3	5·78	+	5·32	+	5·25	+	lost
4	5·46	+	5·20	+	5·17	+	5·17
5	5·05	−	...	−	...	+	5·10
6	4·70	−	...	−	...	−	...
7	4·56	−	...	−	...	−	...
8	4·38	−	...	−	...	−	...
9	4·28	−	...	−	...	−	...

Experiment No. 12 (Laboratory Experiment R. S. 15). Formic acid.

Data (see also Fig. 7).

Series A. Inoculated with D 82 (ninety-six hours old on milk).

Series B. Inoculated with D 83 (ninety-six hours old on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tube to ferment was No. 5, Series B, with value of $P_H = 5{\cdot}06$.

The next set of tubes, with $P_H = 4{\cdot}77$, failed to ferment.

TABLE XV. (*Exp.* 12.)

		Series A		Series B	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·44	+	5·45	+	5·45
2	6·10	+	5·43	+	5·53
3	5·74	+	5·39	+	5·33
4	5·42	+	5·23	+	5·22
5	5·06	−	...	+	5·15
6	4·77	−	...	−	...
7	4·60	−	...	−	...
8	4·44	−	...	−	...

Experiment No. 13 (Laboratory Experiment R. S. 14). Acetic acid.

Data (see also Fig. 8).

Series A. Inoculated with D 81 (forty-eight hours old on milk).

Series B. Inoculated with D 80 (forty-eight hours old on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tubes to ferment were Nos. 4, Series A and B, with value of $P_H = 5{\cdot}21$.

The next set of tubes, with $P_H = 4{\cdot}95$, failed to ferment.

TABLE XVI. (*Exp.* 13.)

		Series A		Series B	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·52	+	5·39	+	5·40
2	5·98	+	5·37	+	5·35
3	5·56	+	5·30	+	5·29
4	5·21	+	5·29	+	5·10
5	4·95	−	...	−	...
6	4·81	−	...	−	...
7	4·75	−	...	−	...
8	4·65	−	...	−	...
9	4·60	−	...	−	...

Experiment No. 14 (Laboratory Experiment R. S. 10). Butyric acid.

Data (see also Fig. 8).

Series A. Inoculated with D 74 (forty-eight hours old on cooked meat).

Series B. Inoculated with D 75 (1) (forty-eight hours old on milk).

Series C. Inoculated with D 75 (2) (forty-eight hours old on milk).

The tubes were examined ninety-six hours after inoculation.

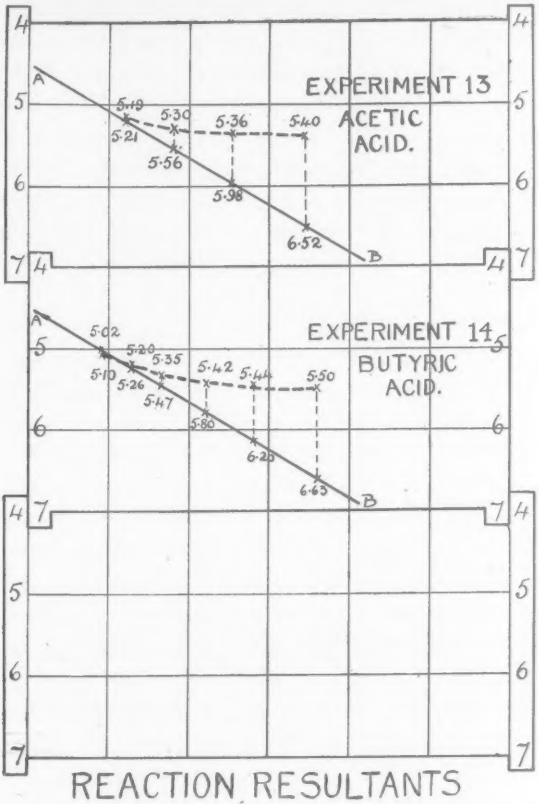


Fig. 8

TABLE XVII. (*Exp.* 14.)

No. of tube	Initial reaction, P_H	Series A		Series B		Series C	
		Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·63	+	5·51	+	5·45	+	5·52
2	6·20	+	5·46	+	5·37	+	lost
3	5·80	+	5·49	+	5·39	+	5·48
4	5·47	+	5·42	+	5·35	+	5·23
5	5·26	+	5·17	+	5·18	+	5·34
6	5·02	+	5·15	+	5·10	+	5·08
7	4·90	−	...	−	...	−	...
8	4·80	−	...	−	...	−	...
9	4·76	−	...	−	...	−	...

The most acid tubes to ferment were Nos. 6, Series A, B and C with value of $P_H = 5{\cdot}02$.

The next set of tubes, with $P_H = 4{\cdot}90$, did not ferment.

When inoculated into glucose peptone media at different reactions *B. sporogenes* behaves similarly to *B. perfringens*. In the first place the higher the acidity of the reaction the longer is the latent period before obvious signs of growth are seen. There also seems to be a fairly well defined critical reaction at which growth is just permitted, as can be seen from the table below.

TABLE XVIII.

Experiment No.	Acid	Most acid value of P_H which allows growth	Least acid value of P_H which inhibits growth	Mean value of P_H. Critical P_H
10	Hydrochloric	4·99	4·75	4·87
11	Lactic	5·05	4·70	4·87
12	Formic	5·06	4·77	4·91
13	Acetic	5·21	4·95	5·08
14	Butyric	5·02	4·90	4·96

Mean value of critical $P_H = 4{\cdot}94$

This critical reaction, $P_H = 4{\cdot}94$, is very close to the value obtained for *B. perfringens*. These two organisms, *B. sporogenes* and *B. perfringens*, are widely different in character and in chemical behaviour, yet they are both equally affected by the initial hydrogen ion concentration of the medium into which they are implanted.

When the final reactions of *B. sporogenes* are considered, the matter is found to be complicated by the formation of both acids and bases. In a previous paper we have shown that fermentation by *B. sporogenes* is accompanied by the production of large amounts of ammonia in addition to acids. This naturally affects the final reaction so that one is unable to draw such definite conclusions from the reaction resultants as could be done in the case of *B. perfringens*.

The reaction resultants, however, show a similar orderly relation between the initial and final reactions. This is the more surprising when one considers the complicated character of the fermentations. In spite of the formation of several distinct acids, large amounts of amino-acids and ammonia, all having a great influence on reaction either as sources of H-ions or OH-ions, or merely as buffers, this orderly reaction persists. Obviously the various phases of fermentation must be very definitely inter-related in order that this nicely balanced state of affairs should exist.

It will be observed that the reaction resultants in most cases cross the line *AB*, the hydroxyl ions of the ammonia actually causing a decrease in the acidity of the medium after a certain stage in the fermentation is reached. To this is no doubt due the ability of *B. sporogenes* to ferment for a very long period. The cessation of activity is probably caused almost entirely by the accumulation of the products of metabolism, and very little by the inhibiting influence of hydrogen ions.

Summary.

An investigation has been made on the effect of acids on the growth of *Bacillus perfringens* and *Bacillus sporogenes*. This was preceded by an examination of the behaviour of the acids used with nutrient media.

The addition of acids to liquids containing large amounts of buffer substances—to which category all efficient nutrient media belong—produces a complex effect on the true reaction of the media. This effect is partly determined by the type of acid employed and partly by the nature and content of buffer substances in the mixture. With the less highly dissociated acids a point is soon reached where successive amounts of added acid affect the reaction but slightly.

The action of acids on the growth of the bacteria in question can be divided into three parts.

The first effect noticed is on the latent period of growth. The more highly acid the medium, the greater time elapses before obvious signs of growth are observed.

The second effect is on the final reaction at which growth ceases. While the limits for the cessation of growth with all acids are comparatively narrow, the differences observed are distinct and obviously governed by definite laws. They are quite wide enough apart to negative the idea of a "physiological constant." The final reaction is undoubtedly dependent on the initial reaction. With a given acid, and varying initial concentrations, a series of final reactions is obtained which can be expressed by a curve. Each acid shows a curve which is individual and to this extent we may speak of specificity of acid effect. The difference between the acids is not large.

The third feature of the action of acids on these bacteria is the total inhibition of growth.

This varies for *Bacillus perfringens* between $P_H = 4{\cdot}99$ using butyric acid and $P_H = 4{\cdot}66$ using malic acid, and has an average value of $P_H = 4{\cdot}82$,

which we have called the critical reaction. This, for practical purposes, may be considered the inhibiting reaction.

It will be observed that for *B. perfringens* the hydrogen ion concentration for inhibition may be lower than that reached by the medium at the end of fermentation. That is to say, after overcoming the inhibiting influence of a certain hydrogen ion concentration (as shown by the lengthened latent period) the bacillus causes a rise in concentration of hydrogen ion by producing acids. During fermentation a secondary toxic effect is observed. This is due to the accumulation of metabolic products, and to the influence of the anions and the undissociated molecules of the acid initially present. Fermentation therefore ceases when different amounts of acid have been formed. The final hydrogen ion concentration also varies. When a highly dissociated acid has been used to produce the initial reaction, and the high concentration of hydrogen ion has been produced by the addition of a small quantity of acid, the acid and gas production is little affected. This is shown in Experiment 9, where hydrochloric acid was used for this purpose. With feebly dissociated acids, much larger amounts are necessary to produce the same hydrogen ion concentration, and the acid exerts an effect which is not only due to the cation. The *mass* of the acid molecule inhibits fermentation, and smaller amounts of acid fermentation products are formed. Hence the toxic action of a weak organic acid is twofold.

With *B. sporogenes*, inhibition takes place at an average value of $P_H = 4{\cdot}94$. This organism differs from *B. perfringens* in that the hydrogen ion concentration of the fermented medium may be lower than that of the medium before inoculation, although large amounts of acid have been formed. Obvious signs of growth may cease to be observed at a concentration of hydrogen ions which is less than the critical reaction. This is to be explained by the complex character of the katabolism of this organism whereby large amounts of basic and amphoteric substances are produced and neutralise the acids, as well as acting as buffers.

Stoppage of growth is probably due almost entirely to the accumulation of metabolites. The tendency of *B. sporogenes* to produce a constant or even lower hydrogen ion concentration than that observed at the time of inoculation may account for the fact that fermentations with *B. sporogenes* may continue for very long periods.

In conclusion one may say that the fermentation of both these organisms is inhibited by a rise in hydrogen ion concentration. The acidity may merely delay growth, or it may stop it entirely. The effect of all the acids we have

used in the course of these experiments is alike. Specific qualities are almost entirely absent.

These experiments seem to show that by submitting the organisms involved in gas gangrene infections to solutions of a hydrogen ion concentration slightly above the critical point growth may be inhibited. They afford confirmation of the views expressed in our first paper that the treatment of local infections of this type by means of acid solutions, highly buffered, is worthy of trial.

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XXII. THE NITROGENOUS EXTRACTIVES OF TUMOURS.

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During the course of an investigation of the proteins of tumours, the results of which have already been reported by the author [1916], the protein-free aqueous extracts of a large number of tumours were obtained. In view of the scanty data recorded in the literature dealing with the extractive substances found in tumours, it was decided to make an examination of this material. The preparation of the extracts was carried out along the usual lines. The fresh tumour tissues in a finely minced condition were extracted with boiling distilled water, a trace of acetic acid being added to complete the removal of coagulable proteins. To the filtered extracts basic lead acetate was added until no further precipitation occurred, the resulting precipitate being filtered off, well washed, and the excess of lead removed from the filtrate by sulphuretted hydrogen.

The filtrate from the lead sulphide, freed from H_2S by passage of a rapid current of air, was acidified with sulphuric acid to the extent of 5 %, and the basic substances precipitated by the addition of a slight excess of a 30 % solution of phosphotungstic acid. After standing overnight in the refrigerator room the precipitate was filtered off and well washed with 5 % sulphuric acid. Each such precipitate was dried in a desiccator over sulphuric acid and stored in this form until sufficient material of each class had been collected for examination.

The filtrates from the phosphotungstic acid precipitations were quantitatively freed from the excess of the precipitating reagent and sulphuric acid by means of baryta, filtered and evaporated to small bulk by distillation *in vacuo* at 40°. In this concentrated form the fractions were stored at 0°, until sufficient had been collected.

The tumours examined were a number of human tumours of mammary origin, a lymphosarcoma, a large secondary deposit in the liver from a pancreatic carcinoma and a series of the avian neoplasm known as the Rous chicken sarcoma.

I. Extractive Nitrogen of the Rous Chicken Sarcoma.

By the method just described it was found possible during the course of several months to collect fractions from the aqueous extracts of 4320 g. of this tumour.

The tumours were grown by the inoculation of grafts of the tumour into the pectoral muscle of healthy, adult, pure bred, fowls of the Plymouth Rock strain. The resulting tumours grow rapidly, infiltrating and destroying the adjacent muscle tissue, so that after some three weeks' growth the tumour has usually attained the size of a hen's egg and weighs 100 g. or more. Under conditions which result in a very rapid rate of growth, such as implantation into young birds, the tumours usually undergo extensive central degeneration during the latter stages, and the cavity of the growth becomes filled with a thick, mucoid, blood-stained fluid.

I (*a*). *Phosphotungstate Precipitate.*

The combined precipitates of phosphotungstates from the extracts of the 4320 g. of tumour tissue weighed in the dry condition 225 g. After being ground to a fine state of division and passed through a fine mesh sieve, it was decomposed in aqueous suspension by means of a hot concentrated solution of baryta. The clear filtrate was quantitatively freed from the excess of barium by sulphuric acid and evaporated down by distillation under reduced temperature and pressure to a thick syrup. The residue was submitted to extraction with hot alcohol, thus removing 5·2 g. of a dirty white substance, insoluble in that solvent and mainly inorganic in nature. The alcohol was removed from the combined extracts by distillation and the residue taken up in a litre of distilled water. This solution was then fractionated by the use of silver nitrate and baryta into four fractions:

I (*a*) i. Purine fraction.
I (*a*) ii. Histidine fraction.
I (*a*) iii. Arginine fraction.
I (*a*) iv. Lysine fraction.

The last fraction consisted of the re-precipitated phosphotungstates after the separation of the first three fractions and the subsequent removal of the excess of silver and baryta.

I (*a*) i. *Purine Fraction.*

The purine silver compounds were decomposed in aqueous suspension by means of sulphuretted hydrogen gas and the silver-free filtrate concentrated down to dryness at reduced temperature and pressure.

The resulting dirty white residue was dissolved in a small volume of dilute hydrochloric acid, filtered and rendered alkaline with ammonia. On standing, a very small quantity of a dirty granular product separated out. Insufficient was obtained to permit of satisfactory purification and analysis, but its reactions, and the isolation of the crystals of the hydrochloride and phosphotungstate, were sufficient to identify the substance as guanine. After neutralisation of the filtrate and the addition of picric acid solution, a crop of crystals of adenine picrate was obtained. Recrystallised from water, 0·7 g. of the purified picrate was isolated (M.P. 273°). Analysis by means of the nitron method for the estimation of picric acid gave the figures:

0·0264 g. adenine picrate; 0·0372 g. nitron picrate; 59·5 % picric acid.

Calculated for $C_5H_5N_5$, $C_6H_3O_7N_3$, H_2O ... 60·0 % „ „

The filtrate from the adenine picrate, freed from the excess of picric acid by acidification with nitric acid and extraction with toluene, was evaporated almost to dryness, when typical crystals of hypoxanthine nitrate separated out. After recrystallisation from dilute nitric acid, 0·4 g. of the pure salt was obtained. No xanthine or other substance was isolated from this fraction.

I (*a*) ii. *Histidine Fraction.*

The silver compounds were decomposed in aqueous suspension by H_2S gas, and the neutralised, silver-free filtrate evaporated to dryness at reduced temperature and pressure. The small residue was extracted with hot alcohol, thus removing a small amount of insoluble inorganic salts, and the alcoholic extracts were freed from alcohol under reduced pressure. The residue gave positive reactions for histidine. Accordingly it was dissolved in 100 cc. of distilled water, and mercuric sulphate solution added until no further precipitation occurred. The small mercury precipitate was filtered off and decomposed in aqueous suspension by H_2S gas, the filtrate quantitatively freed from sulphuric acid and evaporated down on the water-bath with dilute hydrochloric acid. After standing for several days in the desiccator, a very small yield of the typical crystals of histidine hydrochloride was obtained, which, after one recrystallisation from dilute alcohol, melted at 251°. From the hydrochloride the picrolonate was prepared, twice recrystallised from hot

water, and analysed by the micro-Dumas method of Pregl, after being dried *in vacuo* at 110° for 2 hours (M.P. 224°).

3·57 mg.; 0·695 cc. N gas (19°, 766 mm.) 23·2 % N

Calculated for $C_6H_9O_2N_3$, $C_{10}H_8O_5N_4$ ... 23·45 % N

Although there was evidence of the presence of other substances in the histidine mother liquors, attempts to isolate these in a pure condition failed.

I (*a*) iii. *Arginine Fraction.*

The silver salts were decomposed in the usual manner in aqueous suspension with H_2S gas, and the silver-free filtrate evaporated down to small bulk at reduced temperature and pressure. No crystalline product could be isolated direct, but after the addition of a solution of picrolonic acid and on standing some days, a small amount of a micro-crystalline picrolonate was obtained. After recrystallisation from hot water 0·12 g. of the salt was yielded, which showed a melting point of 255° and gave, on analysis, figures which indicated that it was probably an impure preparation of arginine picrolonate. Attempts to purify the salt and analyse the purer product were unsuccessful.

The filtrate from this picrolonate was freed from the excess of picrolonic acid by extraction of the acidified solution with ether and concentrated down to a small bulk. On the addition of a saturated aqueous solution of picric acid a very small crop of a pale yellow crystalline picrate was deposited. After one recrystallisation from water the picrate melted at 214°, and was identified as creatinine picrate by the reactions of the hydrochloride prepared from the picrate. The occurrence of creatinine in this fraction is probably due to its being carried down during the precipitation of the silver compounds.

I (*a*) iv. *Lysine Fraction.*

The reprecipitated phosphotungstates comprising this fraction were decomposed in the usual manner by means of baryta, the excess of barium quantitatively removed, and the filtrate concentrated *in vacuo* at reduced temperature. Hot alcohol extraction of the resulting syrup left behind a considerable amount of insoluble inorganic salts. The hot alcohol extracts on cooling deposited a crop of a pure white crystalline substance, which was filtered off and recrystallised from hot alcohol. The purified substance separated out in clusters of needles and showed a melting point of 244°. Its reactions corresponded with those of carnosine, and its identity with that substance was established by the isolation of the characteristic copper salt, and the analysis of the free base.

0·0589 g. (dried 110° *in vacuo*); 10·20 cc. 0·1 *N* NH_3 ... 24·2 % N
Calculated for $C_9H_{14}O_3N_4$ ... ... 24·6 % N

After separation of the carnosine, it was found that the alcoholic filtrate gave a copious crystalline precipitate with alcoholic picric acid. The picrate after standing overnight at 0°, was filtered off and recrystallised from water, 16 g. of the pure compound being thus obtained. The product was readily identified as creatinine picrate, M.P. 213°.

0·0765 mg. of the picrate, dried at 110° for 2 hours *in vacuo*;

0·1205 mg. of nitron picrate ... ... 66·7 % picric acid.
Calculated for $C_4H_7ON_3$, $C_6H_3O_7N_3$ ... 67·0 % „ „

The hydrochloride prepared from the picric acid salt melted at 264°, and gave all the reactions for creatinine hydrochloride.

No other substance was isolated from this fraction except a small yield of what was probably impure leucine.

I (*b*). *Phosphotungstic Acid Filtrate.*

The concentrated fractions, prepared as already described, had been stored in the refrigerator room and upon examination it was found that a considerable crystalline deposit had separated out. This consisted mainly of tyrosine, but typical crystals of leucine were also identified as being present. After recrystallisation 0·6 g. of fairly pure tyrosine was obtained and recognised by its reactions.

The filtrate from the tyrosine was concentrated to a thick syrup under reduced temperature and pressure, another small yield of tyrosine being obtained during the concentration, and taken up in hot alcohol.

The alcoholic extracts gave a heavy crystalline precipitate on the addition of alcoholic picric acid. The deep yellow picrate, recrystallised from water, showed an imperfect melting point at 236—250°, and was identified as creatinine potassium picrate.

0·0970 g.; 0·1731 g. of nitron picrate ... 75·5 % picric acid.
0·0953 g.; 0·1702 g. „ „ ... 75·6 % „ „
Calculated for $C_4H_7ON_3$, K, $(C_6H_3O_7N_3)_2$ 75·3 % „ „

Creatinine hydrochloride, with a melting point of 258°, was prepared from the double picrate.

Attempts to separate other substances in a pure condition from this fraction failed, although several impure products were isolated, of which one was apparently an impure specimen of leucine, whilst another was probably a still more impure preparation of glycine.

II. Extractive Nitrogen of Mammary Carcinomata.

The chief difficulty encountered in this investigation was the collection of sufficient material. Altogether, some 600 g. of mammary tumour tissue were available, but it was not all of the same type, as was the large amount of chicken tumour used in the first section of the work. The majority of the specimens—weighing together about 450 g.—were primary carcinomata of the mammary gland. Of these 75 % were of the slow growing scirrhus type, the remainder being more or less encephaloid in character. The remaining 150 g. of tissue examined was made up of carcinomatous deposits in the axillary glands, secondary to primary growths in the breasts, and one myxo-sarcoma of the same organ. The combined aqueous extracts of these tissues were treated by the process described in the first portion of this paper.

II (*a*) *Phosphotungstate Precipitate.*

The combined phosphotungstate precipitates weighed only 35 g. They were reduced to a fine state of division and decomposed in aqueous suspension by means of baryta. The neutralised filtrate was then sub-fractionated, as described in the first section of this paper (p. 247), into four fractions.

Owing to the fractions being so very small, the separation of substances in a pure condition from them was rendered exceedingly difficult.

From the purine fraction II (*a*) i, after an attempt at purification of the substances present by the copper bisulphite process, a very small yield of adenine picrate was obtained, M.P. 270°, but no other substance was isolated.

The second and third fractions, II (*a*), ii and iii, although worked up as carefully as possible, yielded no pure products. A substance giving a strong coloration with diazo-benzenesulphonic acid, and precipitated by mercuric sulphate, was detected in the histidine fraction; whilst from the arginine fraction a small yield of a very impure picrolonate was obtained, but in both cases the amount was insufficient to permit of identification.

From the lysine fraction, treated in a similar manner to the corresponding fraction from the Rous extract, a small yield of creatinine picrate was isolated. After purification 0·2 g. of the salt was obtained, M.P. 216°, from which a hydrochloride was prepared, showing the typical reactions and a M.P. of 261°. From the creatinine mother liquors a very small yield of a picrate melting at 180—190° was isolated, but the quantity was too small to permit of purification or identification.

II (*b*). *Phosphotungstic Acid Filtrates.*

From the phosphotungstic acid filtrates, decomposed and stored as already described, a small yield of tyrosine had separated out. This was removed, the filtrate evaporated to dryness and the residue taken up in alcohol. On the addition of alcoholic picric acid, and standing, a very small yield of creatinine picrate in a fairly pure condition was obtained. This was identified by its melting point, 214°, and by the reactions of the free base prepared from the picric acid salt. No other substance was isolated in a pure condition from this fraction.

III. Extractive Nitrogen of a Lymphosarcoma.

This tumour, weighing some 900 g., was an exceedingly rapidly growing sarcoma of the cervical glands occurring in a young man. The growth was firm and showed little evidence of necrotic change having occurred. The aqueous extract of 800 g. of the tumour was examined.

III (*a*). *Phosphotungstic Acid Precipitate.*

This precipitate weighed 29 g. in the dry state, and was decomposed in the usual manner. The resulting solution of bases was fractionated by the silver method.

III (*a*) i. *Purine Fraction.*

This was the largest silver fraction. It was decomposed and the purine bases reprecipitated by the copper bisulphite process. From the solution of the purine hydrochlorides, obtained by decomposition of the copper compounds, ammonia precipitated 0·02 g. guanine. This was identified by its reactions and the crystalline form of its hydrochloride. From the guanine filtrate picric acid precipitated a fairly heavy yield of adenine picrate. After one recrystallisation, 0·42 g. of the picrate, melting at 272°, was obtained. From the oxy-purine fraction a very small yield of impure xanthine was separated, but no evidence of the presence of hypoxanthine was obtained.

III (*a*) ii *and* iii. *Histidine and Arginine Fractions.*

These fractions were very small and no pure substance was isolated from them.

III (*a*) iv. *Lysine Fraction.*

This fraction was worked up in a similar manner to other lysine fractions, but only a small yield of creatinine in the form of its picrate (0·2 g., M.P. 214°) and traces of tyrosine and leucine were isolated.

III (*b*). *Phosphotungstic Acid Filtrate.*

From this fraction 0·12 g. tyrosine and 0·65 g. leucine were separated in a fairly pure condition.

IV. Extractive Nitrogen of Carcinoma of Pancreas.

The material for this examination was a mass of secondary deposits in the liver from a primary carcinoma of the pancreas. The primary growth weighed but 29 g., whilst nearly 5 kilos. of tumour tissue were obtained from the liver deposits. These growths had undergone somewhat extensive central necrosis, with the formation of caseous material. The aqueous protein-free extract of 3·5 kilos. of this material, excluding as far as possible the more extensively degenerated areas, was examined.

IV (*a*) i. *Purine Fraction.*

This fraction was fairly large and was submitted to an initial purification by the copper bisulphite process. Guanine was isolated in very small amount, 0·07 g. of the somewhat impure base being precipitated by ammonia. This was identified in the usual manner. From the guanine filtrate picric acid threw down a small precipitate of adenine picrate. After recrystallisation 0·25 g. of the purified salt was obtained (m.p. 275°).

After removal of the picric acid from the adenine filtrate the oxy-purines were reprecipitated by the copper bisulphite procedure.

From the decomposed copper compounds 0·03 g. of xanthine and 0·06 g. of hypoxanthine nitrate were isolated by the usual methods.

IV (*a*) ii *and* iii. *Histidine and Arginine Fraction.*

These fractions were very small in quantity and no substances were isolated in a pure condition from them. Indications of the presence of histidine were observed, but the amount present was too small to permit of its isolation or identification.

IV (*a*) iv. *Lysine Fraction.*

The reprecipitated phosphotungstates constituting this fraction weighed in the dry state 27 g. The solution of free bases obtained by decomposition of this precipitate gave upon concentration and treatment with picric acid solution a small yield of creatinine picrate. After one recrystallisation 0·37 g. of the pure picrate was obtained (m.p. 217°).

After freeing the solution from picric acid and further concentrating, a small yield (0·07 g.) of tyrosine was isolated in a fairly pure condition. Traces

of impure leucine were also separated from the residue, which, however, was largely inorganic in nature.

IV (*b*). *Phosphotungstic Acid Filtrate.*

Leucine (0·44 g.) and tyrosine (0·27 g.) were isolated from the concentrated filtrate of this fraction. From the mother liquors picric acid precipitated a further small yield of creatinine picrate (0·11 g. purified salt, M.P. 214°).

No other substance was isolated from this extract.

Discussion of Results.

The results of the four examinations recorded above agree with the scanty published data on tumour extractives. Saiki [1909] examined a mixed batch of tumours, consisting of human carcinomata from various sources, and weighing in all some 200 g. and found 0·001 % of purine nitrogen in the protein-free aqueous extracts. He was unable to isolate the individual purines owing to the small amount of material at his disposal. In another trial he found that the purine bases present in 570 g. of mixed cancerous tissue, mainly carcinomata of the ovary, were as follows: 0·1391 g. uric acid, 0·1866 g. adenine, 0·0190 g. hypoxanthine, and traces of guanine and xanthine. He suggests that these observations indicate the presence of guanase and xanthoxidase, and the absence of adenase in human tumours.

Wells and Long [1913] found that the purine content and purine enzymes of human tumours, both malignant and benign, resembled those of normal tissues, and that xanthoxidase was absent. They found it difficult to explain the high percentage of uric acid found by Saiki, since the latter author gives practically no details of the methods employed in the isolation or identification.

Saiki also records finding creatinine to the extent of 0·016 % in fresh cancer tissue, and very small amounts of creatine.

The presence of considerable amounts of carnosine and creatinine in the extract of the Rous chicken sarcoma may be attributed to some extent to the extensive degradation of muscle tissue consequent on the tumour invasion.

Autolysis is probably responsible for the presence of the amino-acids, for Beebe [1904] found leucine, tyrosine, glycine and tryptophan in tumour extracts.

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XXIII. A STUDY OF THE WATER-SOLUBLE ACCESSORY GROWTH PROMOTING SUBSTANCE IN YEAST. I.

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The researches of Stepp [1909, 1912, 1913], Hopkins [1912] and Funk [1913, 1, 2, 3] have demonstrated the importance of certain constituents of a normal dietary which for want of better names have been variously termed "vitamines," growth substances, and accessory growth hormones. It is now known that unless an adequate supply of these as yet unidentified substances is present in the food supply of an animal, serious consequences affecting the general nutrition of the subject will arise. Recently McCollum and Davis [1915, 1, 2, 3] have carried out some researches which have yielded results indicating that two distinct types of accessory substance exist. These by reason of their properties have been conveniently designated the "fat-soluble *A*" and the "water-soluble *B*." The view held by these authors that both substances are equally important components of a normal dietary has received experimental confirmation by Drummond [1916], and has been accepted by the majority of other investigators [Macallum, 1916; Osborne and Mendel, 1916]. Following up his studies along these lines McCollum, in collaboration with Kennedy [1916], has arrived at the conclusion that the production of polyneuritis in birds by exclusive rice feeding [Eykmann, 1897; Funk, 1913, 1, 2, 3], or exclusive feeding of a ration made up of purified foodstuffs [Funk, 1914, 1] is a specific result of the absence of the water-soluble factor *B*. In other words it is proposed to regard the so-called "antineuritic vitamine" as being identical with the water-soluble factor *B*. McCollum and Kennedy have based this suggestion upon the curative value possessed by many extracts, known to contain the water-soluble substance, when administered to birds which have developed the typical symptoms of polyneuritis gallinarum (Eykmann), as a result of feeding solely upon a diet lacking in that substance.

Our knowledge of the chemistry of these accessory substances or "vitamines" is as yet lamentably small, so that should further experimental evidence be forthcoming in support of the proposal advanced by McCollum and Kennedy it would mark a distinct advance in this field of research.

For some time past an investigation has been in progress in this laboratory aiming at the isolation and identification of the dietary factor *B*.

Viewed from this standpoint alone the results of the research have been disappointing, but on the other hand many observations have been made during its progress which help to throw a certain amount of light upon the nature of this elusive substance.

Taken collectively these observations tend to uphold the view advanced by McCollum and Kennedy, since it is found that the chemical properties of the water-soluble growth promoting factor *B* are, as far as they have been studied, very similar to those which have been described for the antineuritic principle. Ever since Eykmann demonstrated that the condition induced in fowls by a diet of polished rice was analogous to human beri-beri, and that there existed a substance in the layers of the rice grain removed during polishing which would cure the condition, numerous investigators have attempted to isolate the curative agent in a pure state.

From the mass of literature which records the results of these many attempts it is difficult to abstract that which may be regarded as sound. Various properties of the curative substance have been described at one time or another. Chamberlain and Vedder [1911] showed that it was soluble in alcohol and dialysable, whilst Cooper and Funk [1911] demonstrated that it would withstand drastic acid hydrolysis and that it was precipitated by phosphotungstic acid.

Since these facts seemed to point to the active substance being of the nature of a comparatively simply constituted nitrogenous base, several investigators attempted to isolate the pure substance along the lines usually adopted in such cases. Funk [1911] confirmed that the substance was precipitated by phosphotungstic acid, and made the further observation that upon sub-fractionation of the substances precipitated by this reagent by the silver process it was to be found in association with the pyrimidine bases. These results were repeated and confirmed by him [1912, 1], and upon these facts he based his reasons for assigning to the product the name "vitamine" [1912, 2]. At about the same time several isolations of the active substance were reported [Schaumann, 1914; Edie, Evans, Moore, Simpson and Webster, 1912; Susuki, Shinamura and Odake, 1912].

The last three claims, however, have now been shown to be unfounded [Funk, 1913, 1], whilst the crystalline derivatives isolated by Funk himself from the pyrimidine fraction of an alcoholic extract of rice polishings, and thought by him to be active [1912, 1], were shown to consist of impure specimens of nicotinic acid and betaïne [Drummond and Funk, 1914]. Pol [1917] has recently described the isolation of a crystalline acid from *Phaseolus radiatus* which he states has cured polyneuritis in birds and beri-beri in man. The details of his process have, unfortunately, not been available, but it is improbable that the crystalline substance he has isolated is the curative substance in a pure condition, because the author mentions that the preparation loses its activity as purification progresses. It is conceivably the same type of product as was isolated by Funk, where a definite crystalline body (nicotinic acid) in some cases contained the active substance as a contamination.

We are therefore practically without knowledge regarding the chemical nature of the antineuritic substance and cannot claim that much progress has been made in obtaining such information during the past few years.

With regard to the properties of the substance itself we are in possession of a number of scattered observations which have been made from time to time, and which generally speaking are in agreement.

The antineuritic substance is apparently only partially soluble in alcohol. This solubility is very small in absolute alcohol, but becomes greater as more dilute alcohol is used [Funk, 1912, 1; Cooper, 1913, 1; Vedder and Williams, 1913; Sullivan and Voegtlin, 1916].

It is usually described as being insoluble in ether, chloroform and other lipoid solvents [Cooper, 1913, 1]. It is adsorbed by animal charcoal [Chamberlain and Vedder, 1911; Cooper, 1913, 1] whilst a colloidal aluminium silicate (Lloyd's reagent) has recently been used for quantitatively adsorbing the antineuritic substance [Seidell, 1916; Eddy, 1916].

Most authors agree that the substance is unstable under certain conditions to alkali. Cooper [1913, 1] reported its destruction by that agency, and this observation has been confirmed [Chamberlain, Vedder and Williams, 1912; Fraser and Stanton, 1915; Funk, 1915]. On the other hand Williams and Seidell [1916], are inclined to doubt whether cold alkali does exert an adverse influence upon the substance, whilst Steenbock [1917] definitely states that only hot alkali has such an effect.

With regard to the effect of acids, most authors agree that it is stable even when they are applied hot and in a relatively concentrated condition. Cooper

and Funk [1911] found the curative value of yeast extracts unaffected by hydrolysis for twenty-four hours with 20 % sulphuric acid, and this stability to acid is confirmed in papers by Funk [1911], Vedder and Williams [1913], Williams and Seidell [1916], and Steenbock [1917]. The degree of thermostability possessed by the substance has been noted by several investigators who appear to agree that temperatures below 100° do not exert a deleterious effect [Cooper and Funk, 1911; Steenbock, 1917]. Higher temperatures, however, are usually considered to injure its activity.

The value of yeast as an antineuritic has been recorded upon several occasions [Cooper and Funk, 1911; Funk, 1912, 1; Cooper, 1913, 2; Schaumann, 1914; Willcox, 1916; Seidell, 1916].

As has already been remarked the first named authors showed that acid hydrolysis failed to lower the antineuritic value of yeast, whilst Cooper [1914] has shown that autolysed yeast is as active as was the original yeast.

Barsickow [1913] found yeast itself to be curative, but that heated yeast and "cerolin" (a yeast extract) possessed no such value. He believed that the curative action was due to the action of nuclein or certain salts.

Funk [1913, 1] investigated the curative value of yeast- and thymus-nucleic acids and some of their degradation products when administered to polyneuritic pigeons, and found that certain of these substances exerted a beneficial influence upon the progress of the disease. Some of the many attempts to isolate the active substance from yeast preparations have already been alluded to. More recent ones are those described by Seidell [1916], and Williams and Seidell [1916].

Attention must now be directed to a consideration of the recorded properties of the water-soluble growth promoting accessory factor. That yeast possessed a rich supply of a growth promoting factor was noted by Hopkins [1912] and by Funk [1913, 2; 1914, 2]. The latter author has since then made several attempts to isolate the substance along lines such as were employed by him in his efforts to isolate the antineuritic substance from the same source.

These attempts have failed to achieve their object, but his results show that the active substance possesses several properties, also possessed by what he terms the "anti-beri-beri vitamine." Funk and Macallum [1915] confirmed the presence of the growth promoting substance in dried brewer's yeast, and in a later paper [1916] they described its presence in autolysed yeast and recorded that it is precipitated by phosphotungstic acid. Attempts at further fractionation of the resulting precipitate failed, for no growth

promoting powers were possessed by the purine or pyrimidine fractions obtained from it. Funk [1915, 1916] has stated that the growth promoting accessory from yeast forms a phosphotungstate insoluble in acetone.

With regard to the solubility of the growth promoting accessory *B* it is known that it is extractable by alcohol [Stepp, 1909; Hopkins, 1912; and others]. It is stable to temperatures below 100° [Drummond, 1916], but is apparently to some extent adversely affected by autoclaving at higher temperatures [Hogan, 1917]. From this brief summary, which is scarcely a complete review of the literature describing the two substances, it will be seen that many of the properties by which the antineuritic substance is described are also possessed by the water-soluble growth accessory factor.

As has already been remarked McCollum and Kennedy have suggested the identity of the two substances [1916], and the same view is apparently held by Macallum [1916].

The experiments which are reported in this paper entirely support this view and make it almost certain that the two substances are either identical or are individually closely related members of the same class of substance.

Experimental[1].

For the purposes of this research young rats from five to six weeks old and weighing approximately 50 g. were utilised. They were drawn from healthy pure-bred stock reared under careful observation and belonged to the black variety of *Mus norvegicus* [Donaldson, 1915]. This particular strain has been found eminently satisfactory for the studies in growth and nutrition which are being made in this laboratory.

The experimental animals were kept in wooden boxes with sawdust covered floors and provided with warm enclosed sleeping quarters.

They were fed twice daily, unused food from the previous meal being removed from the cage upon each occasion. The artificial dietaries were made up into the convenient form of firm balls weighing approximately 5 g. This forms a rough and ready method of estimating the food consumption of the animals. Fresh drinking water is at all times an essential to the health and welfare of the rat, and was in these experiments renewed at least twice daily, the earthenware drinking pans being scalded out on each occasion.

A basal artificial diet was employed which contained all the necessary

[1] I wish to express my gratitude to Mr A. Chaston Chapman for his kindness in supplying me with the highly pure yeast-nucleic acid used in this work.

components of a normal diet with the exception of the water-soluble accessory substance. This diet was made up as follows:

Caseinogen	18 parts	Salt mixture	5 parts
Starch	40 „	Sucrose	17 „
Agar	5 „	Butter-fat	15 „

The various components were carefully purified before use by extractions designed to remove all trace of the water-soluble growth factor. The composition of the salt mixture together with full details of the preparation and purification of the various components of the basal diet are described in a recent communication by Halliburton and Drummond [1917].

That this diet was for all intents and purposes free from the water-soluble accessory may be seen from Chart I, which may be compared with Charts II and III in which are represented the growth curves of rats fed upon a diet such as the above to which had been added the missing accessory substance in the form of yeast preparations. The animals fed upon these diets showed normal growth and reproduction upon the completed ration.

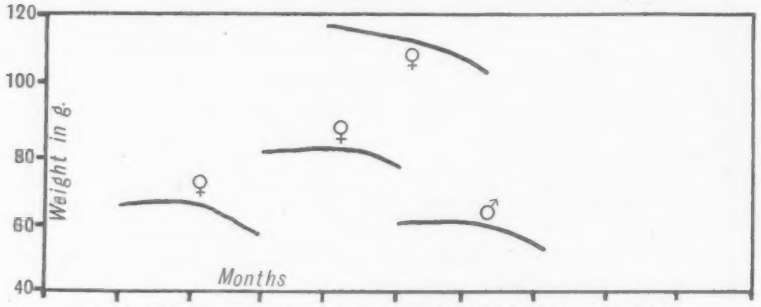

Chart I. Growth curves of rats fed upon basal diet deficient in water-soluble growth promoting accessory factor *B*.

1. *Presence of the water-soluble growth promoting accessory substance in dried yeast and commercial yeast extract.*

Chart II shows the influence upon the growth of young rats exerted by the addition of 3–12 % of dried yeast powder to the basal diet. The rats were able to complete their life cycle without any difficulty, and the animals reared solely upon this diet appeared as healthy and as resistant to disease as those which had enjoyed a normal mixed ration.

Chart III indicates that "marmite," a commercial yeast extract, is rich in the water-soluble accessory factor. The addition of 6 % of this preparation to the basal diet satisfactorily makes good the inadequacy it previously

possessed. Smaller additions of "marmite" were less efficient in this respect, whilst greater percentages were apparently not well tolerated by the animals. These results confirm the findings of the authors that have been referred to with regard to the presence of the water-soluble accessory factor in yeast and its aqueous extract.

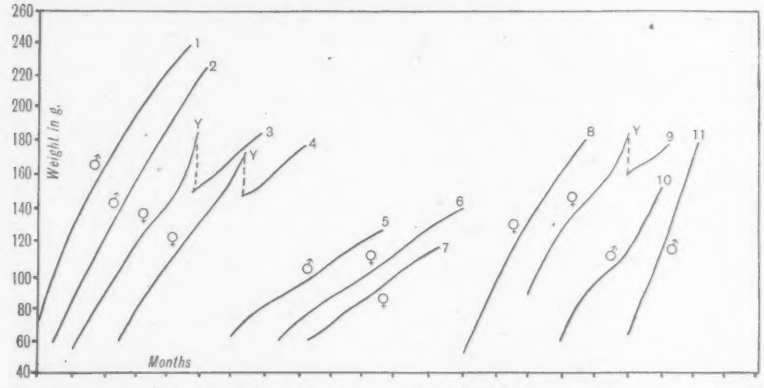

Chart II. Curves 1–4. Growth of rats on basal diet + 6 % dried yeast. Curves 5–7. Growth of rats on basal diet + 3 % dried yeast. Curves 8–11. Growth of rats on basal diet +12 % dried yeast. Y indicates birth of young. These were all reared satisfactorily by the mothers.

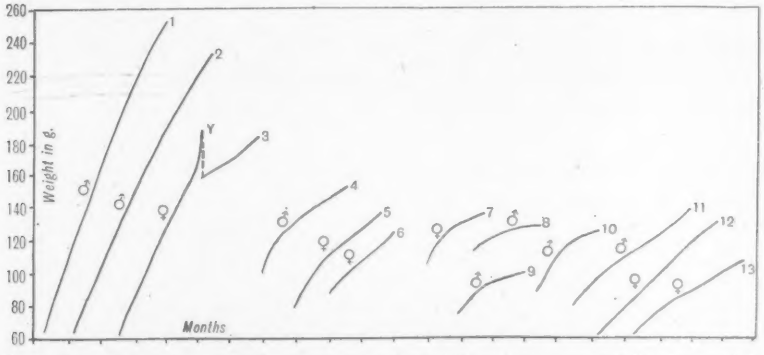

Chart III. Curves 1–3. Growth of rats on basal diet + 6 % "marmite." Curves 4–6. Growth of rats on basal diet + 3 % "marmite." Curves 7–10. Growth of rats on basal diet + 1 % "marmite." Curves 11–13. Growth of rats on basal diet + 12 % "marmite." Y indicates birth of young. All were reared satisfactorily by the mother.

2. *Solubility of the water-soluble accessory substance in alcohol and ether.*

The accessory factor is not removed from dried yeast by extraction with absolute alcohol, so that it appears that the substance is insoluble in pure

alcohol (Chart IV). The dried yeast for this experiment was extracted with several changes of boiling alcohol under reflux. Similarly from the same chart it can also be seen that prolonged ether extraction removes little or none of the active substance.

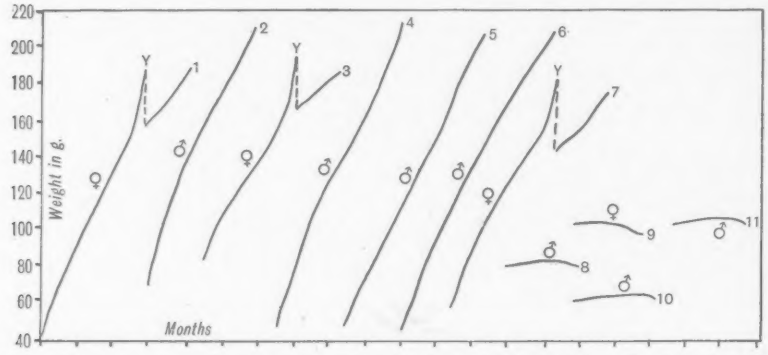

Chart IV. Curves 1–4. Growth of rats fed on basal diet + 6 % alcohol-extracted dried yeast. Curves 5–7. Basal diet + 6 % ether extracted yeast. Curves 8–11. Basal diet + alcohol extract equivalent to 6 % of dried yeast. Y marks birth of young.

It may be mentioned here that dried yeast contains but negligible traces of the fat-soluble growth promoting accessory substance.

Extraction of "marmite" by grinding up with absolute alcohol and repeatedly shaking removes but a small proportion of the accessory factor (Chart V), but when extracted with a more dilute alcohol (70 %) the proportion that goes into solution is greatly increased.

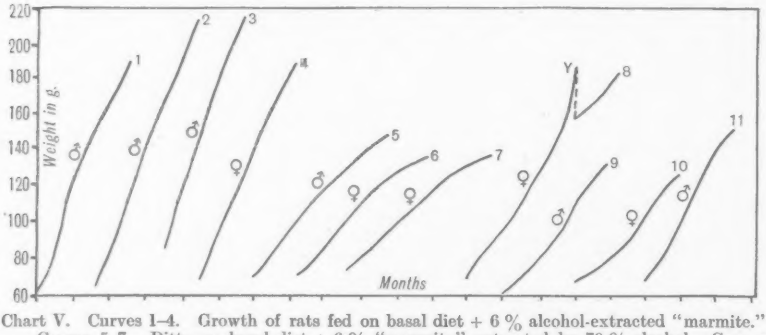

Chart V. Curves 1–4. Growth of rats fed on basal diet + 6 % alcohol-extracted "marmite." Curves 5–7. Ditto on basal diet + 6 % "marmite" extracted by 70 % alcohol. Curves 8–11. Ditto fed on basal diet + alcohol extract equivalent to 6 % "marmite." Y indicates birth of young. All were reared.

3. *Dialysis of yeast extract.*

A strong solution of "marmite" in distilled water was dialysed against running distilled water for forty-eight hours. The combined dialysates were evaporated to small bulk *in vacuo* at low temperature and tested for the presence of the accessory substance under consideration. Chart VI shows that the substance is dialysable through parchment paper, although it was not completely removed from the "marmite" in the time during which the dialysis was carried out. This dialysate contained large amounts of inorganic salts chiefly potassium phosphate and sodium chloride and considerable amounts of leucine and adenine.

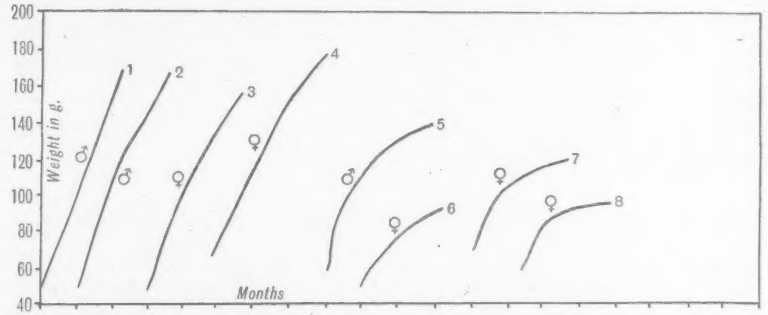

Chart VI. Curves 1–4. Rats fed upon basal diet + dialysate representing 6 % "marmite." Curves 5–8. Rats fed upon basal diet + 6 % residue remaining after dialysis.

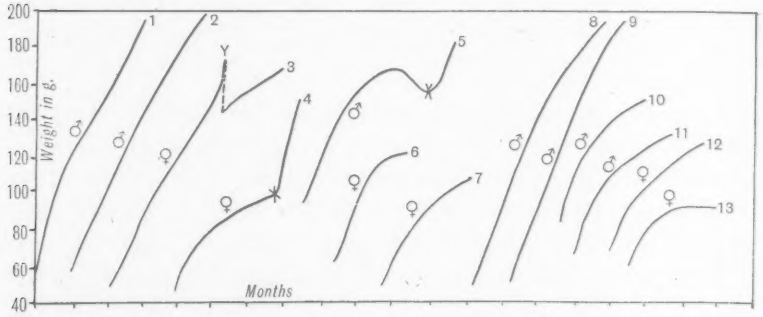

Chart VII. Curves 1–4. Basal diet + 6 % dried yeast heated to 100° for 30 mins. Curves 5–7. Basal diet + 6 % dried yeast autoclaved at 120° for 30 mins. Curves 8 and 9. Basal diet + 6 % "marmite" heated at 100° for 30 mins. Curves 10–13. Basal diet + 6 % "marmite" autoclaved at 120° for 30 mins. Y indicates birth of young. × indicates the time at which the addition of 6 % "marmite" was made to the diet.

4. *Effect of temperature upon the water-soluble growth promoting accessory substance.*

Chart VII demonstrates the growth promoting value of yeast and yeast extracts that have either been subjected to a temperature of 100° or have been autoclaved at 120° for thirty minutes.

The activity of these preparations was practically unaffected by the first treatment, whilst injury but not complete destruction resulted in the second case.

5. *Influence of acids.*

Chart VIII indicates the behaviour of the accessory factor in the presence of acids. One sample of "marmite" was subjected to the influence of dilute hydrochloric acid (1 %) at boiling point for twelve hours, the resulting fluid being carefully neutralised and concentrated to the original bulk. This preparation was not as valuable in promoting growth when added to the basal diet as was a corresponding amount of the untreated marmite, but the activity was not greatly diminished.

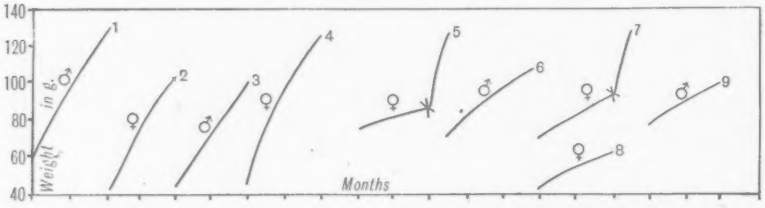

Chart VIII. Curves 1–4. Basal diet + 6 % "marmite" after dilute hydrochloric acid treatment. Curves 5–9. Basal diet + 6 % "marmite" after sulphuric acid hydrolysis. × indicates the time at which the addition of 6% "marmite" to the diet was made.

A second quantity of "marmite" was subjected to hydrolysis with 20 % sulphuric acid at boiling point for ten hours. The sulphuric acid was then quantitatively removed from the diluted liquid by means of baryta, and the neutral filtrate, together with the washings of the bulky precipitate, was evaporated to a thick syrup at a low temperature under diminished pressure.

This preparation possessed a greatly decreased power to promote growth in the experimental animals. In view of the observations which are given later in this paper, this result is regarded as being due not so much to an actual destruction of the accessory substance as to loss occurring by adsorption on the bulky precipitate of barium sulphate and pigmented matter.

6. *Influence of alkali.*

Chart IX displays the growth curves of rats fed upon the basal diet together with additions of treated yeast preparations submitted to various treatments with alkali. One set of curves demonstrates that treatment of "marmite" for twenty-four hours at room temperature with 5 % sodium hydroxide results in very little depreciation in its growth promoting value.

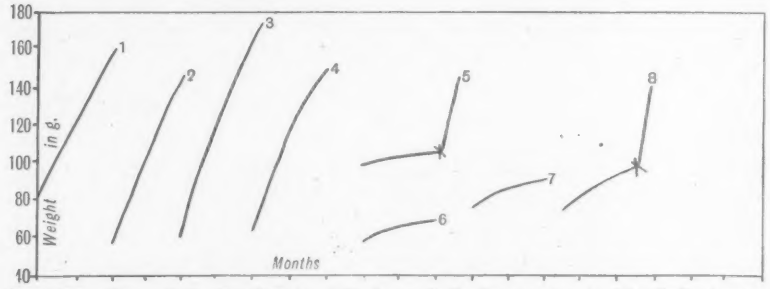

Chart IX. Curves 1–4. Basal diet + 6 % "marmite" after treatment with alkali at room temperature. Curves 5–8. Basal diet + 6 % "marmite" after hot alkali treatment. × indicates the time at which the addition of 6 % "marmite" to the diet was made.

On the other hand treatment with hot 5 % sodium hydroxide for five hours tends to destroy its activity. In both these cases after treatment with alkali the fluid was neutralised and carefully evaporated *in vacuo* at low temperature to its original bulk.

Attempted separation of the active substance.

1. *Precipitation from extracts by phosphotungstic acid.*

A preparation consisting of the concentrated dialysate from 200 g. of commercial yeast extract, prepared as already described, was diluted to a volume of 2000 cc., acidified to the extent of 5 % with sulphuric acid, and precipitated by the addition of a 30 % aqueous solution of phosphotungstic acid. The flocculent, buff-coloured precipitate was allowed to stand overnight at ice-room temperature and filtered off next morning, the precipitate being thoroughly washed with 5 % sulphuric acid. When dry the precipitated phosphotungstates weighed 87 g. Since the majority of such salts contain phosphotungstic acid to the average extent of 85 % of their weight, this yield represents some 12 g. of substances removed from the extract. The precipitated phosphotungstates were decomposed by treatment with

hydrochloric acid and extraction with the amyl alcohol-ether mixture recommended by van Slyke [1915]. Very incomplete decomposition was effected by this method, for a bulky flocculent precipitate, probably representing precipitated pigment, resisted decomposition. The aqueous fraction was separated after repeated extraction, filtered, neutralised and evaporated to small bulk at reduced temperature and pressure.

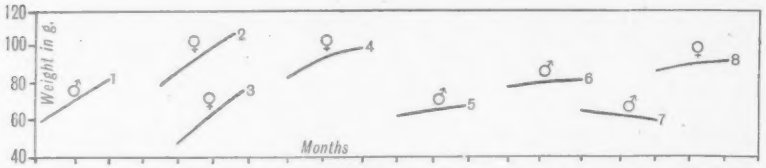

Chart X. Curves 1–4. Basal diet + phosphotungstic acid precipitate fraction corresponding to 18 % "marmite." Curves 5–8. Basal diet + phosphotungstic acid filtrate fraction corresponding to 18 % "marmite."

Chart X demonstrates that this fraction possessed a small growth promoting activity when administered in doses representing 6–18 g. of the original "marmite." Great loss had, however, occurred during the process. The filtrate from the phosphotungstic acid precipitation was freed from sulphuric acid and the excess of the precipitant by means of baryta and after concentration tested for activity. None however was observed (Chart X).

Whether the active substance is completely precipitated by phosphotungstic acid is uncertain, for any which was unprecipitated would be adsorbed and lost during the removal of the sulphuric and phosphotungstic acids by baryta, owing to the very bulky precipitate which results. Great loss also occurred without a doubt due to the adsorption of the active body by the flocculent residue of undecomposed phosphotungstates which has been already noted.

A further preparation of the phosphotungstates from 200 g. of "marmite" was made, and weighed in the dry condition 90 g. This was treated when quite dry by shaking up with repeated changes of acetone [Funk, 1916]. A large proportion (69 g.) of the precipitate remained undissolved. It would seem probable from studies upon the solubilities of the phosphotungstates in acetone (not yet published), that the inference to be drawn from this fact is that the chief extractives of yeast precipitated by phosphotungstic acid are purine bases or the inorganic bases potassium and ammonium. Both fractions of the phosphotungstic acid precipitate were freed from acetone, decomposed by the amyl alcohol-ether process and worked up for inclusion in the diets

as in the former case. A large proportion (40 %) of the acetone-insoluble phosphotungstate was not decomposed by this process, and again formed a flocculent precipitate which much interfered with the separation.

The curves shown in Chart XI demonstrate that the water-soluble growth accessory factor is mainly present in the acetone-insoluble fraction of the phosphotungstic acid precipitate, although great loss has occurred during the separation.

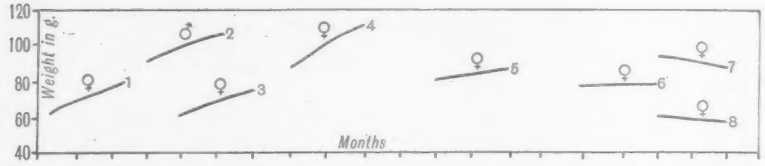

Chart XI. Curves 1–4. Basal diet + acetone-insoluble phosphotungstic acid precipitate fraction corresponding to 10 % "marmite." Curves 5–8. Basal diet + acetone-soluble phosphotungstic acid precipitate fraction corresponding to 10 % "marmite."

2. *Basic lead acetate precipitation of yeast extracts.*

A 10 % solution of "marmite" gave an exceedingly bulky flocculent precipitate upon the addition of basic lead acetate solution. After removing this precipitate and subjecting it to a thorough washing, both precipitate and filtrate were decomposed by sulphuretted hydrogen gas, the lead-free solutions being neutralised and concentrated at low temperature and diminished pressure.

Little growth promoting power was observed in either fraction (Chart XII), it being considered probable that the heavy and flocculent nature of the lead sulphide precipitates had resulted in practically complete adsorption and loss of the active substance.

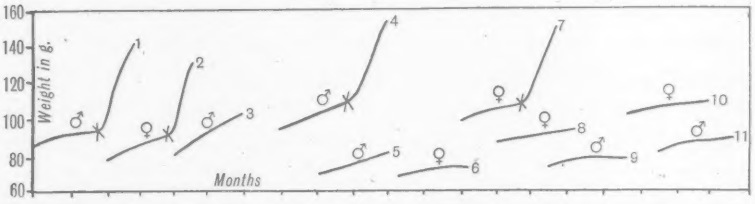

Chart XII. Curves 1–3. Basal diet + lead acetate filtrate fraction corresponding to 6 % "marmite." Curves 4–6. Basal diet + lead acetate precipitate fraction corresponding to 6 % "marmite." Curves 7–9. Basal diet + lead acetate filtrate fraction representing 6 % dialysed "marmite." Curves 10 and 11. Basal diet + lead acetate precipitate fraction representing 6 % dialysed "marmite." × indicates the time at which the addition of 6 % "marmite" to the diet was made.

A sample of the dialysate from the "marmite" was precipitated in a similar manner with basic lead acetate and little evidence of the presence of the active substance in either fraction was obtained, and again great loss had occurred.

3. *Silver nitrate precipitation.*

To the forty-eight hours' dialysate from 250 g. yeast extract silver nitrate was added until no further precipitation occurred. The silver-free filtrate after neutralisation and concentration showed little or no evidence of the presence of the growth accessory substance under investigation (Chart XIII).

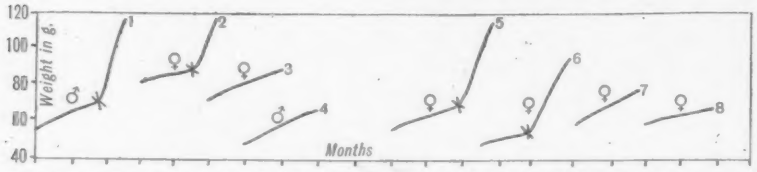

Chart XIII. Curves 1–4. Basal diet + purine fraction from dialysed "marmite" corresponding to 6 % "marmite." Curves 5–8. Basal diet + corresponding pyrimidine fraction. × indicates the time at which the addition of 6% "marmite" to the diet was made.

The silver precipitate was decomposed by sulphuretted hydrogen gas and the filtrate concentrated with care after neutralisation. Small traces of the active substance appeared to be present. Chemical examination of the fraction demonstrated that the majority of the nitrogen was present in the form of adenine.

4. *Relation of adenine to the water-soluble accessory substance.*

Adenine itself was found to possess no growth promoting properties when added to a diet deficient in the water-soluble factor *B* (Chart XIV). This is of interest in view of the fact that the recent work of Williams and Seidell [1916] has not received confirmation [Voegtlin and White, 1916; Steenbock, 1917; Harden and Zilva, 1917]. Williams and Seidell attributed an anti-neuritic action to a supposed isomeric form of adenine. Funk [1913, 1] found a distinct improvement in the condition of polyneuritic pigeons following the administration of thymus-nucleic acid, guanosin, adenosin and cytidine, and slightly less satisfactory results when certain purine and pyrimidine bases were given.

The influence upon growth of pure yeast-nucleic acid and of a nucleic acid prepared from the spleen in a case of spleenic leucaemia was therefore studied, but neither preparation in any way improved the growth value of the basal diet (Chart XIV).

Commercial meat extract ("Lemco") does not show evidence of the presence of the water-soluble accessory factor (Chart XV), so that the identity of the latter substance with any of the meat bases is rendered doubtful. Commercial meat extracts have been found to possess no power to improve the condition of polyneuritic birds [Chick and Hume, 1917].

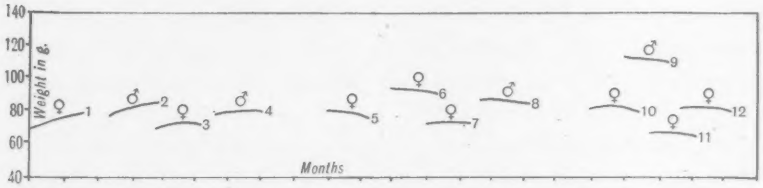

Chart XIV. Curves 1–4. Basal diet + 1 % yeast nucleic acid. Curves 5–8. Basal diet + 1 % spleen nucleic acid. Curves 9 and 10. Basal diet + 0·5 % adenine. Curves 11 and 12. Basal diet + 0·2 % adenine.

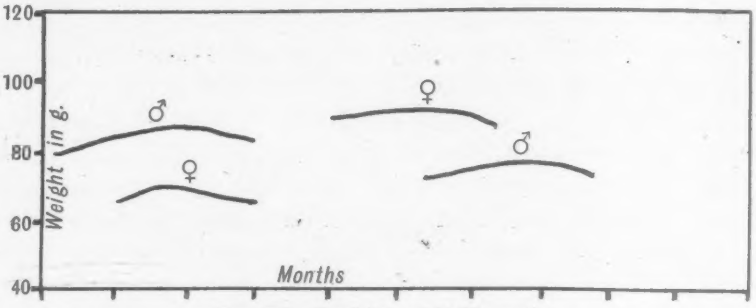

Chart XV. Basal diet +6 % "Lemco."

Discussion.

The results of this investigation, in so far as they are complete, tend to support the view held by McCollum and Kennedy that it is a deficiency of the water-soluble accessory substance in the dietary of birds that is responsible for the incidence of the typical symptoms which are held to represent a pathological condition analogous to beri-beri in man.

Attempts have been made along many lines to isolate the accessory substance from yeast extracts. All of them have however failed to achieve their object, and the only useful result of the investigation which has as yet been obtained has been that a knowledge of a few of the properties of the elusive substance has been gained.

Many workers have lamented the difficulties which are encountered in research in this particular field, and have drawn attention to the way in which the active substances disappear as the fractionation proceeds.

The usual explanation of this has been in the past to regard the substance as unstable. Thus Funk [1913, 3] believed that he had found evidence of the frequent occurrence of nicotinic acid in fractions which had once possessed, but had later lost, curative power, and on these grounds he was inclined to regard that acid as a derivation product of the active "vitamine." Similarly more recent writers have described the existence of isomeric modifications of adenine [Williams and Seidell, 1916], and of certain betaïnes [Williams, 1917], which possess curative properties. This latter work has not, however, received the support of other workers [Voegtlin and White, 1916; Harden and Zilva, 1917], and therefore stands in need of confirmation before it can be accepted.

It seems far more probable that the correct explanation of the loss which occurs during fractionation of extracts containing the antineuritic substance or the water-soluble growth promoting substance is to be found in the readiness with which these substances, whether identical with one another or not, are adsorbed from solution by precipitates.

As has already been mentioned this property was observed several years ago by Chamberlain and Vedder [1911], and Cooper [1913, 1], whilst certain modern work confirms the observation. Thus Seidell [1916] has based a process for separating the substance from autolysed yeast upon its adsorption by fullers' earth, and Voegtlin and White [1916] record that mastic emulsion and colloidal arsenious sulphide will carry down the antineuritic principle in the adsorbed condition.

Further proof that this is probably the explanation of the great loss which is so often encountered in the isolation of the substance is to be found in the fact, that whenever in the course of the fractionation recourse to precipitation is made, the precipitate, particularly if bulky and flocculent in nature, will usually carry down the majority, if not all, of the active substance from solution.

It is naturally a very difficult matter to trace the growth promoting principle during fractionation, because minute traces of that substance do not always demonstrate their presence by causing an appreciable increase in the body weight of the experimental animal, whereas much smaller quantities of such extracts are often sufficient to cause a decided improvement in the condition of a polyneuritic pigeon. Unfortunately neither the experimental results recorded in this paper nor this discussion can throw much light upon

the chemical identity of the class of substance under consideration. One point remains clear, namely, that there is now less evidence than ever to support the continued use of the word "vitamine" coined by Funk. Williams and Seidell [1916] have quite recently supported the adoption of the term, but McCollum and Kennedy [1916] have clearly pointed out that the arguments against its inclusion in biochemical nomenclature far outweigh those advanced by the supporters of Funk, and will continue to do so until our knowledge of the chemical nature of these substances justifies the adoption of the appellation.

Summary.

1. Experimental evidence is given which demonstrates that certain of the properties possessed by the antineuritic substance and the water-soluble accessory growth factor *B*, are very similar.

2. This fact supports the view that the two substances are identical.

3. The repeated failures to isolate substances of this class in the pure condition are attributed more to the fact that they are readily removed from solution in the adsorbed condition by precipitates, than to their reputed instability.

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XXIV. THE REDUCTION OF PHENYL-ETHYLAMINE.

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(*Received August 31st, 1917.*)

The purpose primarily in view was to reduce several compounds closely allied to tyrosine by that method in which the reduction is brought about by shaking the compound in question with hydrogen and a catalyser in the shape of finely divided platinum or palladium. The catalysers were prepared by reducing solutions of the respective chlorides by means of formaldehyde and alkali [Willstätter and Hatt, 1912][1]. A point to be noted is that in all instances mentioned in the course of this paper where reduction did not take place, the catalyser employed was subsequently tested and found to be active. This goes to prove that the negative results in these instances are to be attributed to inherent reasons, rather than to inactivity of the catalyser. The test applied was to shake castor-oil in ethereal solution with hydrogen and the catalyser to be tested. In every case reduction proceeded very rapidly, the white, solid product of reduction simultaneously falling out of solution. Generally ten minutes sufficed for the complete reduction of 5 g. of the oil.

Previous to being subjected to reduction, the hydrochloride of phenylethylamine was purified and tested. This sample of the base had been prepared from phenylalanine by splitting off carbon dioxide. Recrystallised from alcohol, the hydrochloride was found to melt at 215°, thus proving its purity. The compound decolorised permanganate solution instantly. Its chloroplatinate (rhombs and flakes from water) was found to decompose at approximately 250°. The properties of the free base were likewise found to agree with the properties of phenylethylamine in all respects.

Of this hydrochloride, 0·6 g. was reduced by shaking at ordinary temperature in aqueous solution with hydrogen and a catalyser in the shape of finely divided platinum. The absorption of hydrogen proceeded but very slowly, so that the 253 cc. theoretically requisite for complete reduction had been absorbed only at the end of about 1·5 hours. Almost precisely at this point, the absorption stopped entirely. This is a decided proof that no leakage,

[1] Cf. H. Meyer's *Determination of Radicals in Carbon Compounds* (3*d.* original edition) for a detailed description of the method and its application.

but rather solely absorption had been taking place. Another 1·35 g. sample of the same hydrochloride was reduced in the same way. In this case also, the absorption stopped as soon as the requisite volume of hydrogen (570 cc.) had been absorbed. After filtering off the platinum, the solutions were evaporated, thus yielding the *hydrochloride* of the reduction-product in the shape of a white, crystalline mass. This compound was found to be readily soluble in water. It was purified by dissolving it in alcohol, and reprecipitating by means of ether. It melts at 252°, *i.e.* approximately 40° higher than the hydrochloride of unreduced phenylethylamine. The compound no longer decolorises permanganate solution—an additional sign that complete reduction has taken place. Millon's reagent causes a white precipitate which however readily redissolves when gently heated.

The chloroplatinate. With platinic chloride, a voluminous precipitate resulted. This salt was always obtained finely crystalline on recrystallising it from water. It decomposes and chars at 255–257°, previously becoming somewhat darker at 220°. While the chloroplatinate was being decomposed by magnesium, during the chlorine determination, a very strong basic odour became apparent.

0·1362 g. vac.-dry; 0·0000 g. H_2O; 0·0399 g. Pt; 0·1751 g. AgCl.
0·1363 g. vac.-dry; 0·0000 g. H_2O; 0·0398 g. Pt.

	Found		Calculated for hexahydrophenylethylamine chloroplatinate $(C_8H_{17}NHCl)_2PtCl_4$
Pt	29·29 %	29·20 %	29·40 %
Cl	31·80		32·04

The aurichloride. Recrystallised from water containing a few drops of hydrochloric acid, this salt takes the shape of large, very thin, striated, glistening flakes. It was dried in a vacuum desiccator. It melts somewhat indistinctly at about 135–138°.

0·1275 g.; 0·0000 g. H_2O; 0·0538 g. Au
0·1421 g.; 0·0000 g. H_2O; 0·0599 g. Au
Found 42·23 % 42·19 % Au
Calculated for $C_8H_{18}N$, $AuCl_4$: 42·23 % Au

The picrate. Recrystallised from water, this at times formed prismatic needles, and at other times, transparent, glistening flakes. It was found to melt at 155–156°.

This examination of the reduction-product fully suffices to establish its identity as hexahydrophenylethylamine, for it and its derivatives agree in every respect with the data upon cyclohexylethylamine [Wallach, 1908], as obtained by the reduction of cyclohexylacetonitrile.

An additional attempt to reduce phenylethylamine was undertaken. In contradistinction to that of the two reductions just mentioned, the sample of base now employed represented the purchased synthetic product, in the shape of the hydrochloride. An attempt to reduce 1 g. of this by means of the afore-mentioned method showed that the compound would absorb no hydrogen whatsoever. Before repeating the attempt, the compound in question was thoroughly examined, in order to confirm its identity with phenylethylamine.

The hydrochloride crystallised from alcohol in thin flakes, melting at 214–216°, thus agreeing perfectly with the previous sample. The substance however decolorised permanganate solution but very slowly; a dilute solution requiring several hours. The base, liberated by alkali, represented an oil with an odour much like that of methylamine. The base quickly absorbs carbon dioxide from the air, thus producing a carbonate which forms thin flakes. The base dissolves in ether and in alcohol. The chloroplatinate, recrystallised from water, forms small, faintly yellow flakes, generally lozenge-shaped, joined and superimposed to form spear-like structures. The compound becomes darker at 210°, black at 220°, and then melts at about 255°. It is practically insoluble in alcohol, sparingly soluble in cold water, more readily soluble in hot water. As regards the chloroplatinate, it will be noted that Bernthsen (working with the synthetic base) found it to be more readily soluble in alcohol than in water, whereas Schulze and Barbieri (working with the base from phenylalanine) found it to be far more readily soluble in water. In addition a few minor discrepancies were evidently noted to exist between the bases mentioned. The picrate crystallises from water or alcohol, either in small plates or large, thick prisms, M.P. 169°. The aurichloride is readily formed upon gently heating the mixture of the components. Recrystallised from water acidulated with hydrochloric acid, it forms very thin, shapeless, faintly yellow flakes, M.P. 98–99° (air-dried). Millon's reagent and mercuric chloride form white precipitates with the compound.

According to this examination—as becomes apparent upon consulting the properties quoted for phenylethylamine and its salts—the base in question agrees with phenylethylamine in all respects, and is accordingly identical with it. Practically the sole difference noted between the synthetic base and that obtained from phenylalanine was the slightly differing behaviour towards permanganate solution. In judging this point, it is well to recall that Bernthsen, as opposed to Schulze and Barbieri (as has been pointed out), noted a difference between natural and synthetic phenylethylamine; this

slight difference being evident in the solubility of the respective chloroplatinates. Two additional attempts to reduce the present sample of phenylethylamine were made according to the afore-mentioned method of reduction, and with freshly prepared platinum and palladium catalysers. In neither case was any hydrogen whatsoever absorbed by the compound. For the time being, no explanation can be ventured for this apparent difference between the two samples of phenylethylamine here subjected to reduction.

Attempted reduction of tyrosine. The tyrosine employed was obtained by hydrolysis of Italian silk, by means of sulphuric acid. The purified product, M.P. 301–302°, was subjected to reduction by the above-mentioned method. Six attempts were made under various conditions and with various active catalysers, but in no case was any hydrogen whatsoever absorbed. In working up the tyrosine from these attempts, a small amount of phenylalanine was isolated in the shape of its copper-salt. Precipitated from alcohol by means of water, this salt formed pale blue, minute flakes.

0·1578 g. (air-dry); 0·0129 g. H_2O; 0·0297 g. CuO
0·1357 g. (air-dry); 0·0109 g. H_2O; 0·0256 g. CuO

	Cu	H_2O
Calculated for $(C_9H_{10}O_2N)_2Cu, 2H_2O$:	14·86 %	8·41 %
Found:	15·02	8·18
	15·08	8·04

The amino-acid itself, upon being subjected to an extensive examination, proved itself absolutely identical with *dl*-phenylalanine [Abderhalden, 1911].

An additional attempt was made to reduce pure isolated phenylalanine (obtained from cheese) by this method of reduction, but the result was again negative.

Attempted reduction of p-*hydroxyphenylethylamine.* The *p*-hydroxyphenylethylamine here subjected to treatment by the above method, had been prepared from tyrosine by carefully heating small amounts in a partial vacuum, in order to split off the carbon dioxide. The purified hydrochloride of this base melted at 266–268° [cf. Takaoki Sazaki, 1914, who obtained a hydrochloride of M.P. 268–269°], its picrate at 200°, and all the tests applied agreed with the properties of *p*-hydroxyphenylethylamine. Several attempts to reduce this compound were undertaken, but in no case could reduction be effected.

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XXV. FURTHER OBSERVATIONS ON THE INFLUENCE OF PHENOL AND OF CRESYLIC ACID ON THE CONCENTRATION OF ANTITOXIC SERA BY THE BANZHAF (1913) PROCESS.

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(*Received July 27th, 1917.*)

It has been already pointed out [Homer, 1917] that in the Banzhaf [1913] process for the concentration of antitoxic sera the difficulties encountered in filtration can be obviated by (1) an adjustment of the reaction of the sera, and (2) by the addition of phenol, cresylic acid or "trikresol" to the ammonium sulphate mixtures previous to their being heated.

The first of these methods of procedure requires more care and attention than can always be afforded in a laboratory where concentrations are carried out on a commercial scale. The second is much simpler and gives good results as may be seen from the details of the further experimental work which has been carried out in this connexion.

Phenol and its homologues not only increase the heat denaturation of the serum proteins but also so influence the precipitability of the particles of heat denaturated protein that they are retained with the First Fraction precipitate. This phenomenon, presumably brought about by virtue of the effect of the above mentioned substances on the surface tension of the protein particles in colloidal solution, leads to the production of a final product which is clear and which filters readily through filter candles.

Experience has shown us that, in order to obtain the desired results, the percentage of these substances added to the serum, must fall within certain limits. Should the amount not reach the lower limit then the end products are more unsatisfactory than if no phenol, etc., had been added. Should the upper limit be exceeded then some of the antitoxin is transferred to the First Fraction Precipitate from which it cannot be recovered by extraction with 30 % of saturation with ammonium sulphate; for this purpose prolonged extraction with brine is necessary.

Experimental concentrations illustrating this point, each with not less than one litre of plasma, were carried out as follows:

To oxalated plasma were added 2 % of solid sodium chloride and one-fifth of its volume of water[1]. To the diluted plasma were then added varying amounts of phenol or of cresylic acid followed by the volume of a saturated solution of ammonium sulphate necessary to bring the ammonium sulphate content of the mixture to 30 % of saturation.

The phenol or cresylic acid-serum-ammonium sulphate mixtures were distributed in stoppered glass jars and placed in a bath containing water at a temperature of from 62 to 64°. The temperature of the serum mixtures gradually rose to 60°. As soon as this was reached the jars were taken from the bath and allowed to cool to 45° and filtered (First Fraction Precipitate).

The precipitates were washed with a solution containing ammonium sulphate to the extent of 30 % of saturation. The filtered washings were added to the main bulk of the filtrates which were then brought up to 50 % of saturation with ammonium sulphate. The ensuing precipitate (Second Fraction Precipitate) was filtered, pressed and dialysed in the usual way. To the residue from dialysis was added 1 % of sodium chloride and 0·35 % of cresylic acid.

A comparison was then made between the various end products thus obtained as regards their clearness and their tendency to become cloudy on standing. At the same time data were furnished as to the percentage removal of the total serum proteins and also as to the percentage of the total antitoxic units of the original serum appearing in the end product in those cases in which the higher percentages of phenol and cresylic acid had been used. The antitoxic content of the end products, where lower percentages of these substances had been used, were not estimated in the published series of experiments owing to our shortage of experimental animals. From other work we have satisfied ourselves that in the latter cases there is no appreciable loss of antitoxin during the process of concentration.

The results which have been embodied in the accompanying table (p. 281) indicate that:

[1] Banzhaf recommends that the plasma be diluted with one half its volume of water previous to the addition of the ammonium sulphate. However, in our routine work, we no longer consider the dilution of the plasma of any advantage, for it is our experience that the heat denaturation and precipitation of the serum proteins is a factor of the reaction and not of the dilution of the sera.

In my experimental work the dilution of the plasma with one-fifth its volume of water is a matter of personal convenience for the reading of the scale of the Zeiss Refractometer.

(1) Cresylic acid has a greater effect on the heat denaturation and precipitation of the heat denaturated proteins than has phenol. Moreover, the effect of the former takes place at a lower temperature than that of the latter.

The end products obtained from concentrations in which 0·30 % of cresylic acid had been added to the plasma were more clear and filtered better than when 0·50 % of phenol had been used.

(2) The addition of progressively increasing amounts of phenol or of cresylic acid to samples of the same sera previous to the heating of the serum mixtures was followed by a correspondingly increased percentage removal of the serum proteins of the original plasma.

(3) For the production of clear and readily filterable end products which would remain clear after filtration it was found necessary to add a minimum of 0·30 % of cresylic acid to the diluted plasma.

With a smaller amount of cresylic acid it was found that the end products were cloudy and far more unsatisfactory than if the Banzhaf process had been carried out without the addition of this substance.

(4) While the percentage of cresylic acid may be increased to 0·35 %, it is inadvisable to go beyond this limit, as the ensuing increased denaturation induces a transference of antitoxin to the First Fraction Precipitate from which it can only be recovered by long extraction with brine.

(5) The degree of concentration is considerably increased by the addition of 0·30 to 0·35 % of cresylic acid to the plasma previous to concentration.

Thus, whilst by the Banzhaf (1913) technique during the process of concentration, there is usually a 4 to 5 times increase in potency per cc., with the above modification the potency per cc. is increased 7 to 8·5 times[1].

In view of the above experimental data the concentration of routine batches of 50 and 100 litres of sera was conducted in the same way and similar results ensued. The protocol of the concentration of a 50 litre batch of antitetanic serum is given below.

T. R. 65.

Volume of oxalated plasma taken	50 litres
Volume of water added	10 „
Weight of NaCl added	1200 g.
Volume of cresylic acid shaken with 1 litre of water and added to the diluted plasma	180 cc.
Volume of a saturated solution of ammonium sulphate added	25,700 cc.

[1] The potency can be still further increased (viz. to the order of 10 times) by making the precipitation limit for the Second Fraction Precipitate 45 per cent. of saturation with ammonium sulphate instead of 50 per cent. as hitherto used [Homer, 1917].

Temperature to which the cresylic-plasma-ammonium sulphate mixture was just heated ... ... 60°

Percentage increased precipitation of the serum proteins induced during the heating of the mixtures to 60° 28

Original Plasma	Final Product
Volume, 50 litres	Volume, 6·5 litres
Potency per cc., 200 (slightly +)[1]	Potency per cc., 1650
Protein content, 8·26 %	Protein content, 18·6 %

We thus see that, in the above concentration:

(1) there has been a removal of 70 % of the total proteins of the sera taken for concentration,

(2) the loss of antitoxic units during the process has been negligible,

(3) the potency of the serum has been increased eight times.

The final product was clear and limpid. On dilution with saline it showed no trace of the presence of a colloidal suspension of euglobulin or of heat denaturated protein. Its filtration through filter candles proceeded with ease and the filtered product showed no signs of becoming cloudy on standing.

From these results it will be seen that, by the addition of 0·30 to 0·35 % of cresylic acid to the diluted plasma before concentration by the Banzhaf method, the degree of concentration and the percentage removal of the serum proteins thereby induced are of the same order as those obtained by the method recently published from these laboratories [Homer, 1916]. Such a simple modification of the technique of the former process entailing the production of a highly concentrated product will probably be welcomed by those laboratories in which my double heating process has not yet been adopted.

Summary.

The difficulties encountered in the Banzhaf (1913) technique and the tendency of the end products to become cloudy can be obviated by the addition of 2 % of sodium chloride and of 0·30 to 0·35 % of cresylic acid to the plasma previous to the heating of the plasma-ammonium sulphate mixtures.

There will not be undue loss of antitoxin in presence of the specified amounts of cresylic acid provided that:

(*a*) the addition of 2 % of sodium chloride be made a matter of routine,

[1] The experimental guinea-pig to which 100 M.L.D.'s of U.S.A. Standard Tetanus Toxin and 1/2000 cc. of the above plasma had been given died within 120 to 136 hours after the injection.

A comparison between the end products obtained from the concentration of antitoxic sera by the Banzhaf (1913) *Process and by a modification of the same process in which to the plasma diluted with one-fifth its volume of water have been added varying amounts of phenol and of cresylic acid previous to the heating of the serum mixtures.*

Experimental No.	Percentage of protein in the original plasma	Percentage of phenol or of cresylic acid added to the *diluted* plasma	Percentage increase in the precipitation of the soluble proteins during the raising of the temperature of the phenol or cresylic acid-serum-ammonium sulphate mixtures to 55°	Percentage increase in the precipitation of the soluble proteins during the raising of the temperature of the phenol or cresylic acid-serum-ammonium sulphate mixtures to 60°	Percentage of the total proteins of the original plasma appearing in the final product	Percentage of the total antitoxic units of the original plasma appearing in the final product	Increased potency per cc. over that of the original plasma	Appearance of the final product standing in bulk in the cold room within (*a*) 24 hours, and (*b*) three months of being taken from the dialysing bags: (*a*) after 24 hours	(*b*) after 3 months	The effect of dilution of the end product with twice its volume of saline
Antidiphtheritic serum										
49 *a*	7·81	0·00 phenol	2·1	5·2	64·5	—	—	slightly turbid	turbid	opalescent
49	7·81	0·35 „	11·0	17·0	45·0	>84*	4·5	„ „	„	slightly opalescent
50	7·81	0·50 „	15·5	20·8	40·0	>83*	5·5	very slightly turbid	slightly turbid	very slightly opalescent
51	7·81	0·35 cresylic acid	24·6	26·0	30·0	82	7·5	clear	clear	clear
Antitetanic serum										
75 *a*	6·49	0·00 cresylic acid	—	2·1	65·0	—	—	slightly turbid	turbid	opalescent
75	6·49	0·15 „ „	—	15·0	45·0	—	—	„ „	„	„
80	6·49	0·20 „ „	—	17·0	36·7	—	—	„ „	„	slightly opalescent
81	6·49	0·25 „ „	—	22·5	35·0	88·0	6·5	„ „	slightly turbid	„ „
82	6·49	0·30 „ „	—	25·0	28·1	88·0	8·5	clear	clear	clear
Antidiphtheritic serum										
85	7·61	0·00 cresylic acid	—	3·0	63·1	—	—	clear	turbid	opalescent
86	7·61	0·25 „ „	—	26·0	42·9	—	—	turbid	„	„
87	7·61	0·30 „ „	—	30·0	37·1	—	—	clear	clear	clear

* Not tested further on account of the shortage of experimental guinea pigs. For the same reason tests were not carried out on 49 *a*, 75 *a*, 75, 80, 85, 86, and 87 as we have (in other work) satisfied ourselves that with as much as 0·5 and 0·35 % of cresylic acid there is no undue loss of antitoxin provided that the temperature of the serum mixture be not raised beyond 60°.

(*b*) the mixtures be neither heated beyond 60° nor allowed to remain at this temperature for more than two or three minutes.

The addition of the cresylic acid has considerably enhanced the degree of concentration that can be obtained by the Banzhaf method. It has also increased the percentage removal of the serum proteins.

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XXVI. A NOTE ON THE USE OF INDICATORS FOR THE COLORIMETRIC DETERMINATION OF THE HYDROGEN ION CONCENTRATION OF SERA.

By ANNIE HOMER.

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During the progress of a series of investigations on the concentration of antitoxic sera it was found necessary to adjust the reaction of the sera to certain fixed values in order to get concordant and consistent results. In the experimental work, owing to lack of data with regard to the reliability of indicators in the presence of the serum proteins, the hydrogen ion concentration of the sera was determined by the electrical method. Since the latter method cannot be conveniently used in a general routine laboratory, experimental work was undertaken to ascertain whether determinations of sufficient accuracy for practical purposes could be made by the colorimetric method.

In this connexion to several samples of different sera were added varying quantities of acid or of alkali. The hydrogen ion concentration [H·] of each of the samples was estimated by the electrical method and the values thus found were compared with those obtained by the colorimetric method using the stated indicators and Sörensen's standard solutions in a Walpole's colorimeter, the colour system being as follows:

Eye.

Left	Right
10 cc. serum + *x* drops of Indicator	10 cc. serum + *no* Indicator
10 cc. water + *no* Indicator	10 cc. Sörensen's standard solution + *x* drops of Indicator

Source of Light.

In the colorimetric determination of the values for the [H·] care must be taken to use a constant amount of the specific indicator. It is a matter of experience that, both with the standard solutions and with the test sera, the intensity of the colour reaction at a definite hydrogen ion concentration can be varied by alterations in the amount of indicator added, so that, even with indicators which may prove sufficiently reliable for use with sera, unless the amount of indicator be kept constant, neither consistent nor comparable results will be forthcoming.

For my purpose litmus, methyl violet, methyl orange and Henderson and Forbes' indicator were useless with serum as I was unable to match the two colour systems. In these cases the colour reaction between the serum and each of the above-mentioned indicators seemed to me to be different from that between the indicator and the standard solutions.

With *p-nitrophenol* and *tropaeolin OOO*. it was found that, although it was possible to match the two colour systems, the colour changes were neither sufficiently sharp nor in sufficient contrast to the colour of the serum itself for me to obtain satisfactory readings.

With *phenolphthalein* the colorimetric determinations gave values not sufficiently near those obtained by the electrical method to be of any service even for approximate values for routine work. Thus, the colour reaction between the indicator and serum corresponding to that with the standard solutions at P_H 8·25 was not evidenced until the true reaction of the serum had been brought to values of the order of P_H 10·5.

On the other hand, contrary to expectation, *neutral red* proved sufficiently reliable for routine determinations with sera. It was found that with the addition of five drops (Pasteur capillary pipette) of a 0·05 % solution of this indicator in alcohol to 10 cc. of the Sörensen's standard solutions the useful range was between P_H 5·6 and 7·8. The same amount of indicator was then added to 10 cc. of samples of the different sera and the colour reaction thus produced was matched with the colour scheme obtained by viewing the standard solutions and indicator through serum in the colorimeter: in serum the useful range was between P_H 5·9 and 7·6. The results thus obtained for the values of the [H·] of the sera together with those obtained by the electrical method have been embodied in Table I.

TABLE I.

A comparison between the values obtained for the hydrogen ion concentration (P_H) of various sera by the electrical method and by the colorimetric method, using neutral red as the indicator.

[To 10 cc. of the test solutions were added five drops of a 0·05 % solution of neutral red in alcohol.]

Plasma No.	Percentage of protein in the sample of plasma taken	P_H of the plasma determined by the colorimetric method	P_H of the plasma determined by the electrical method
1	6·73	7·6	7·7
1 *a*	4·92	7·14	7·14
2	6·16	6·98	7·02
2 *a*	4·62	between 6·98 and 6·81	6·82
2 *b*	4·62	6·60	6·40
3	6·16	5·91	6·29
4	8·12	7·35	7·46
4 *a*	6·09	between 7·64 and 7·35	7·50
4 *b*	4·06	7·34	7·40
5	6·09	5·91	6·02
5 *a*	4·06	5·91	6·02
6	6·56	7·34	7·40
6 *a*	6·56	5·90	5·92
6 *b*	4·92	6·97	6·98
6 *c*	4·92	6·60	6·40

A study of Table I shows that the [H·] of sera can be determined between P_H 5·9 and 7·6 with sufficient accuracy for practical purposes by the colorimetric method using neutral red as indicator.

The studies of Sörensen and Palitzch [1910] have shown the value of *α-naphtholphthalein* as an indicator. It was tried with sera as its useful range, using three drops of a 0·10 % solution with the standard solutions, was from P_H 7·6 to 8·5.

From the data given in Table II it will be seen that the determinations by the electrical method are consistently higher than those by the colorimetric method using this indicator. Thus, the slightest tinge of green colorimetrically corresponding to that for P_H 7·6 with the standard solutions does not show until the true value for P_H is of the order of 8·5. However, with this indicator the "buffer" action of the proteins is not sufficiently marked to be too serious a drawback to its use for routine work. Thus, it was found that, if to 10 cc. of the serum containing three drops of the solution of α-naphtholphthalein

ammonia was added until the appearance of the faintest green tinge in the serum was detected, the reaction of the serum thus treated was approximately P_H 8·5.

TABLE II.

A comparison between the values for the P_H of various sera obtained by the electrical method and by the colorimetric method, using α-naphtholphthalein as the indicator.

[To 10 cc. of the test solutions were added three drops of a 0·10 % solution of the indicator in alcohol.]

Plasma No.	Percentage of protein in the plasma	P_H determined by the colorimetric method	P_H determined by the electrical method
7	6·73	7·64	8·29
7 *a*	4·92	7·64	8·04
8	8·12	7·86	8·65
8 *a*	6·09	7·64	8·44
8 *b*	4·06	7·64	8·55
8 *c*	2·03	7·65	8·60
9	6·56	7·64	8·27
9 *a*	4·92	7·64	8·48
9 *b*	4·92	8·04	8·71
10	6·16	7·64	8·52
10 *a*	4·62	7·64	8·45
10 *b*	4·62	7·86	8·25

For routine work in which a value of about P_H 8·0 was desired it was found that the following procedure would approximately give the desired result: To an aliquot part of the serum was added the necessary volume (x cc.) of a standard solution of ammonia to bring the [H·] to the value P_H 7·6, using neutral red as the indicator. To a similar volume of serum was added the necessary volume (y cc.) of the ammonia solution to bring the [H·] to the value P_H 8·5 (faint green tinge with α-naphtholphthalein).

The addition of $\frac{x+y}{2}$ cc. of the standard solution of ammonia to the stated volume of serum brought the [H·] approximately to the value of P_H 8·0. This is a purely empirical arrangement which has given results of sufficient accuracy for routine work for the reason that, while an excess of alkali is undesirable, slight variations in the reaction round about P_H 8·0 are not followed by the marked variations in the heat denaturation of the serum proteins so characteristic of sera more acid than P_H 5·5.

It was next desired to find reliable indicators for the determination of the [H·] of sera more acid than could be tested by neutral red. For this purpose,

owing to the unreliability of litmus, methyl orange, methyl red, Henderson and Forbes' indicator, and also to my inability to match the colour reaction with *p*-nitrophenol, resource was had to the sulphonephthalein indicators, the use of which has been so strongly advocated by Mansfield Clark and Lubs [1915]. The brilliancy of the colour reactions of these substances suggested that besides possibly furnishing indicators for the desired acid ranges they might also replace the indicators already in use on the alkaline side.

In this connexion, the following indicators in 0·05 % alcoholic solution, were used.

	Useful range P_H
Thymol-sulphonephthalein*	8·0–9·6
o-Cresol-sulphonephthalein*	7·2–8·8
Phenol-sulphonephthalein	6·8–8·4
Dibromothymol-sulphonephthalein*	6·0–7·6
Dibromo-*o*-cresol-sulphonephthalein*	5·2–6·8
Methyl red	4·4–6·0
Tetrabromophenol-sulphonephthalein	3·0–4·6

* Samples of these indicators were very kindly sent to me by Dr Mansfield Clark as some delay was experienced in obtaining the chemicals necessary for their preparation.

The useful range of each indicator was tested against Sörensen's standard solutions which were also checked by the electrical method. A comparison was then made between the values of the [H·] of the various sera obtained by the colorimetric method using the above mentioned indicators and also by the electrical method. The data with regard to these observations have been embodied in Table III.

Thymol-sulphonephthalein was found to be unreliable with serum. Thus, the colour reaction corresponding to P_H 8·3 with the standard solutions did not appear in the serum until the value P_H 10·3 had been reached.

The colour changes with *o-cresol-sulphonephthalein* in serum could not be detected by me with sufficient ease for its adoption in routine work.

The colour reactions of *phenol-sulphonephthalein* and of *bromothymol-sulphonephthalein* in serum could be readily matched throughout their useful ranges with the standard solutions, but, the values for the [H·] of the sera thus determined were consistently lower than the true values determined by the electrical method. As it was found that, in sera containing from 4 to 10 % of protein, these deviations from the true values were constant for the same worker, use was made of these indicators for the determination of the [H·] of sera between the ranges P_H 6·8 and 9·0 as follows.

To the test serum were added six drops of a 0·05 % solution of the indicator. The colour reaction was then matched against standard solutions containing

TABLE III.

A comparison between the values obtained for the P_H of various sera by the electrical method and by the colorimetric method using the indicators of the sulphonephthalein series.

Plasma No.	Percentage of protein in the plasma	Indicator used	P_H of the plasma determined by the colorimetric method	P_H of the plasma determined by the electric method
11	8·12	Thymol-sulphonephthalein	8·03	10·34
12	6·16	" "	8·03	10·21
13	6·56	" "	8·03	10·33
14	4·40	" "	8·03	10·55
15	6·73	Phenol-sulphonephthalein	6·81	7·65
15	6·73	" "	7·14	8·00
15	6·73	" "	7·34	8·25
15	6·73	" "	7·64	8·55
15	6·73	" "	7·86	8·70
15	6·73	" "	8·03	8·97
16	4·92	" "	7·64	8·43
17	6·16	" "	7·64	8·46
18	8·12	" "	7·64	8·25
19	6·09	" "	7·86	8·70
20	4·06	" "	7·44	8·27
20 *a*	4·06	Bromothymol-sulphonephthalein	6·23	6·86
21	6·09	" "	6·46	7·31
22	4·92	" "	6·46	7·58
23	4·40	" "	6·46	7·38
24	8·12	" "	6·81	7·86
24	8·12	" "	7·10	8·10
24	8·12	" "	7·30	8·66
25	6·97	" "	7·30	8·60
26	9·90	" "	7·30	8·70
27	6·73	Methyl red	4·98	4·00
28	4·92	"	6·00	5·24
29	8·12	"	5·60	4·80
30	8·12	Bromo-*o*-cresol-sulphonephthalein	5·2	5·14
31	6·09	" "	5·2	5·06
32	6·56	" "	5·2	5·14
33	4·40	" "	5·2	5·23
34	6·73	" "	5·4	5·50
35	7·56	" "	5·4	5·58
36	8·12	" "	5·4	5·65
37	6·56	" "	5·4	5·32
38	6·73	Bromophenol-sulphonephthalein	3·0	2·90
39	8·12	" "	3·0	2·87

a corresponding amount of indicator, the colour produced with the latter being viewed through serum. The value thus obtained was transmuted to the true value according to the following table:

Phenol-sulphonephthalein		Bromothymol-sulphonephthalein	
Value for P_H found by the colorimetric method	True value for P_H of the serum (approximately)	Value for P_H found by the colorimetric method	True value for P_H of the serum (approximately)
6·81	7·65	6·23	6·86
7·14	8·00	6·46	7·40
7·34	8·25	6·81	7·80
7·64	8·55	7·10	8·10
7·86	8·70	7·30	8·60
8·03	8·95		

For the colorimetric work with serum, I found it more easy to match the yellowish green to blue colour reactions of bromothymol-sulphonephthalein than the orange to reddish purple colour reactions of phenol-sulphonephthalein. For this reason in routine work the reaction of sera was approximately adjusted to the value P_H 8·0 by the addition of ammonia until the colour reaction of bromothymol-sulphonephthalein with the serum matched that with a standard solution at P_H 7·1, the latter being viewed through serum in the colorimeter. However, although fairly consistent results were obtained, I found that I was not always as successful with bromothymol-sulphonephthalein as with α-naphtholphthalein, the sensitiveness of my colour vision to the delicate colour changes of the former being very much influenced by personal fatigue.

With *dibromo-o-cresol-sulphonephthalein* and serum it was found somewhat difficult to match the various shades of purple through the ranges of the indicator. However, in practice, it was an easy matter to bring the serum to the degree of acidity at which the indicator showed a slight greenish tinge, *i.e.* just before the acid limit of the useful range of the indicator was reached. The reaction of the sera so adjusted was found to be approximately P_H 5·5.

If acid were added to serum containing bromo-*o*-cresol-sulphonephthalein until the final slight green tinge had just disappeared it was found that approximately the value P_H 5·2 had been reached.

For the adjustment of the sera between the values P_H 5·5 and P_H 4·5 the following arbitrary method was adopted which gave results of sufficient accuracy for routine work, provided the colorimetric determinations were made by the same observer.

Sera containing from 5 to 8 % of protein were brought to P_H 5·5 (circ.) by the addition of the requisite amount (x cc.) of acetic acid to a stated volume of serum containing a fixed amount of indicator until only the faintest tinge of green was detected. Since the colour of serum is affected by its acidity or alkalinity, the comparison in all cases was made with the same volume of serum containing no indicator but to which had been added

a corresponding amount of acetic acid. It was found that the further addition of acetic acid to the serum beyond this point gave results of the following order:

Volume of *N* acetic acid (or equivalent) added to 100 cc. of the serum	P_H of the acidified serum (approximately)
x cc.	5·5
x + 0·5 cc.	5·4
x + 1·0	5·3
x + 1·5	5·2
x + 2·0	5·1
x + 2·5	5·05
x + 3·0	4·9
x + 4·0	4·7
x + 5·0	4·6

The value of such an arbitrary method depends on the acuteness of the colour vision of the individual and, for this reason, in order to obviate the personal error in obtaining the determination of the initial value depending on the colour tint, which, for me, approximated to the value P_H 5·5, it must be urged that each investigator should work out his own table.

Methyl red proved to be unsatisfactory with serum, as the colorimetric determinations gave results not sufficiently near those obtained by the electrical method to justify its use in routine work, slight changes in the reaction of sera on the acid side of P_H 5·5 leading to marked variations in the heat denaturation and precipitation of the serum proteins.

Difficulty was also experienced in matching the purple colour of *bromophenol-sulphonephthalein* in serum. It was however found that, with the sera taken, the faint green tinge just before the useful range of the indicator on the acid side was passed (*i.e.* at P_H 3·0) could be regulated with approximate accuracy.

During the course of the investigation it was found that dilution of the serum was followed by sharper changes in the colour reactions throughout the useful ranges of all the above-mentioned indicators. But, even where the dilution had been of the order of 1 in 4, there was no appreciable variation in the values obtained for P_H by the colorimetric method.

Summary.

During the course of the investigation it has been demonstrated that the hydrogen ion concentration of sera can be determined, with sufficient accuracy for routine work, by the colorimetric method with the use of certain indicators.

It was my experience that:

1. Neutral red gave reliable results with serum throughout the ranges P_H 5·9 to 7·6.

2. The results obtained with α-naphtholphthalein were consistently higher than the true values. Thus, the colorimetric determination of the values for P_H 7·6 was not given until the reaction of the sera had been adjusted to the value P_H 8·5.

3. By a combination of the use of neutral red and of α-naphtholphthalein the adjustment of routine batches of sera to P_H 8·0 can be arranged with sufficient ease for practical purposes.

4. Of the indicators of the sulphonephthalein series thymol-sulphonephthalein and *o*-cresol-sulphonephthalein proved unsatisfactory with serum. Phenol-sulphonephthalein and bromothymol-sulphonephthalein gave results which were consistently higher than the true values. Bromo-*o*-cresol-sulphonephthalein could be used with ease for P_H 5·4 and 5·2 while bromophenol-sulphonephthalein was satisfactory for values about P_H 3·0.

5. By a purely arbitrary arrangement based on my own observations, I was able to adjust the reaction of sera approximately to values between P_H 7·6 and 8·95 with the use of phenol-sulphonephthalein; to values between P_H 6·8 and 8·6 with the use of bromothymol-sulphonephthalein and to values between P_H 5·5 and 4·5 with the use of bromo-*o*-cresol-sulphonephthalein.

6. In view of the personal error involved in the determination of colour reactions with sera it is advisable that each worker using the colorimetric method should ascertain the degree of approximation of his own values to the true values determined by the electrical method.

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XXVII. ON THE INFLUENCE OF THE HEAT DENATURATION OF PSEUDOGLOBULIN AND ALBUMIN ON THE NATURE OF THE PROTEINS APPEARING IN CONCENTRATED ANTITOXIC SERA.

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(*Received Aug. 29th, 1917.*)

In previous communications [Homer, 1916, 1917, 1, 2] it has been demonstrated that, as a result of the heating of serum, considerable changes are effected in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate, the extent of the changes being a function of the reaction of the serum.

The practical application of these results to the concentration of antitoxic sera has led to the preparation of end products (the residue from the dialysis of the protein precipitated between 30 and 50 % of saturation with ammonium sulphate) containing from 20 to 30 % only of the total proteins of the original serum. These results show an improvement on those obtained by the methods hitherto published.

Recent work, however, has shown that the final products obtained by methods involving a heat denaturation of the serum proteins contain albumin as well as pseudoglobulin and, moreover, that the relative amount of albumin increases with the extent of the heat denaturation of the proteins.

The presence of albumin in the final product is to be avoided for two reasons; the one that, since the antitoxin is associated with the pseudoglobulin and not the albumin constituent of the serum proteins, the presence of albumin in the final product must lower the degree of concentration; the other that the inclusion of albumin may be undesirable from the clinical point of view [Dale and Hartley, 1916].

Before attempting to ascertain whether an alteration of the precipitation limits for the Second Fraction precipitate would obviate the precipitation of heat denaturated albumin with the pseudoglobulin, it was necessary to furnish

experimental evidence with regard not only to the extent to which the denaturation of the serum proteins was affected by the time of heating but also to the extent to which the denaturation of the individual serum proteins was affected by the reaction of the serum.

Owing to the necessity for the immediate practical application of the results of the investigation to routine work, the experiments have, for the present, been confined to a study of the behaviour of pseudoglobulin and of albumin in this latter respect.

A. The influence of the time of heating of sera on the extent of the heat denaturation of the serum proteins.

During the progress of the experimental work involved in the study of the influence of the reaction of sera on the extent of the heat denaturation of the serum proteins it was noticed that, with alkaline sera, the changes were a gradual function of the time of heating while, with acid sera, the changes were complete within a comparatively short time.

In order to furnish detailed evidence on this point the following experiments were undertaken. Antitoxic serum to which had been added 2 % of sodium chloride was diluted with one-third its volume of water. To separate volumes, each of 100 cc. of the diluted plasma, were added varying amounts of acid and of alkali and the hydrogen ion concentration [H·] of the various experimental liquids was measured by the electrical method. The stoppered bottles containing the experimental liquids were then placed in a water bath at 57·5° and were heated for several hours at that temperature.

Samples of the experimental sera, previous to their being heated, were made 30 % of saturation with ammonium sulphate and filtered. The protein content of the filtrates was measured by means of the Zeiss refractometer. Similar measurements were made with samples of the heated liquids withdrawn from time to time from the various bottles of sera in the bath. From the data thus obtained was calculated the percentage increase in the precipitability of the serum proteins after the heating of the sera for the stated periods of time.

The results have been incorporated in Table I and indicate that:

(1) During the heating of alkaline sera the extent of the heat denaturation gradually increases with the prolongation of the time of heating.

The maximum value is obtained after four hours' heating at 57·5°.

(2) In the acid sera the extent of the heat denaturation has practically reached its maximum value after one hour at the specified temperature.

(3) The nature of the changes involved in the alkaline sera is different from that in the acid sera, for at no time during the heating of the alkaline sera does acidification of the clear heated liquids to a value P_H 5·0 produce the precipitate of denaturated protein so characteristic of heated acid sera.

During the course of the work it has also been observed that both with acid and alkaline sera the maximum effects are produced:

(*a*) more quickly if the temperature to which the serum is heated be raised beyond 57·5°, and

(*b*) more slowly if the sera be heated at temperatures lower than 57·5°.

TABLE I. *The influence of the time of heating the sera at 57·5° on the extent of the heat denaturation of the serum proteins.*

[Protein content of the plasma = 7·15 %.]

Volume of acid or of alkali added to 100 cc. of the diluted plasma	P_H of the diluted plasma measured by the electrical method	Percentage increase in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate after heating the serum at the specified temperature for a period of				
		1 hr	2 hrs	3 hrs	4 hrs	6 hrs
4 cc. *N* NH_3	9·4	23·1	37·2	—	44·0	45·0
2	9·1	23·0	32·0	—	40·0	40·0
1	8·8	22·2	30·0	—	40·0	40·0
0·6	8·2	17·7	27·0	—	34·6	35·6
0·5 cc. *N* HAc	7·4	12·0	17·0	—	22·0	22·6
0·75	7·1	9·0	11·0	—	16·0	16·8
2·5	5·9	13·0	12·9	13·0	—	—
3·0	5·7	13·0	16·6	16·6	—	—
3·5	5·5	15·0	16·0	16·0	—	16·0
4·0	5·3	20·0	23·5	24·2	—	—
4·5	5·1	30·0	32·8	33·0	—	33·0
5·0	5·0	34·8	40·0	40·0	—	—
6·0	4·8	60·9	67·5	67·0	—	—
6·5	4·7	64·6	79·8	79·8	—	80·0

These results will be of service to those engaged in the concentration of antitoxic sera. They show that, in order to get the maximum effect, in the case of sera more alkaline than P_H 7·4 the time of the preliminary prolonged heating of the serum at 57·5° must not be less than four and need not be greater than six hours after the liquid has reached the specified temperature. In the case of acid sera the full effect is produced within from one to two hours of the temperature reaching 57·5°.

To what extent variations in the temperature at which the serum is heated affect the order of the maximum value for the heat denaturation of the serum proteins at a particular [H·] of the serum is under investigation.

B. THE EFFECT OF CHANGES IN [H·] ON THE DENATURATION OF PSEUDOGLOBULIN INDUCED DURING THE HEATING OF ITS SOLUTIONS AT A TEMPERATURE OF 57·5° FOR A PERIOD OF SIX HOURS.

A solution of pseudoglobulin was prepared as follows:

To four litres of antidiphtheritic serum were added two litres of water and six litres of a saturated solution of ammonium sulphate. The liquid was allowed to stand for one hour and was then filtered. The precipitate was washed with four litres of a half-saturated solution of ammonium sulphate and, after being thoroughly drained, was thrown into four litres of a saturated solution of salt containing an excess of solid salt. The salted liquid was allowed to stand for two days, care being taken to macerate the precipitate thoroughly. The liquid was then filtered and to the filtrate was added 0·25 % of glacial acetic acid. The ensuing precipitate was filtered, pressed and, after the addition of 3 % of solid crystallised sodium carbonate, was dialysed. The residue from dialysis was diluted until the protein content was of the order of 8 %, and 1·5 % of solid salt was then added.

Aliquot parts of the solution were measured into stoppered glass bottles; varying amounts of acid and of alkali were added and the [H·] of each of the liquids was determined by the electrical method. The bottles containing the liquids were then heated in a water bath at a temperature of 57·5° for a period of six hours. The protein content of the unheated liquids and also of the filtrates from samples of the unheated liquids brought up to 30 % of saturation with ammonium sulphate were measured by means of the Zeiss immersion refractometer. The values thus obtained were compared with the corresponding values for the heated liquids. From the data thus furnished were calculated the percentage precipitation of pseudoglobulin itself and also the percentage increased precipitability of the pseudoglobulin by 30 % of saturation with ammonium sulphate induced during the heating of the pseudoglobulin solution. The results which have been incorporated in Table II (p. 297) have also been plotted in Figure 1, Curves 1 and 2.

The appearance of the heated liquids was characteristic throughout the ranges of acidity and alkalinity investigated. On the alkaline side the liquids were clear or were characterised by an opalescence. On the acid side the liquids were converted during the heating into thin suspensions, the semi-solid consistency of which became more marked as the acidity increased.

From a study of the data given in Table II, Column *A*, it will be seen that the conversion of soluble pseudoglobulin into insoluble protein was of the

order of 5 % or less in the liquids of which the reaction lay between the ranges P_H 5·5 and 9·7. On the acid side of P_H 5·5 there was a marked precipitation of insoluble protein; thus, at P_H 5·34 it was of the order of 17·9 % while at P_H 4·6 it was of the order of 78 %.

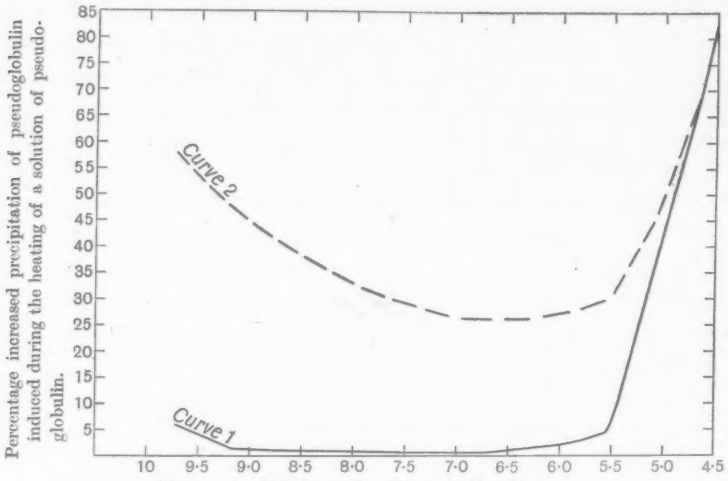

Fig. 1

Curve 1 ——— represents the influence of changes of reaction on the conversion of soluble pseudoglobulin into an insoluble condition during the heating of solutions of pseudoglobulin at 57·5° for 6 hours.

Curve 2 — — — represents the accompanying percentage increased precipitability of the heat-denaturated pseudoglobulin.

The data given in Column *B*, Table II show that there was, throughout the whole range investigated, a marked increase in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate: the extent of the increase was a function of the reaction of the liquid.

The increased precipitability was least between the range P_H 5·5 and 8·0. On either side of these limits the rate of increase was appreciable: the gradient for the increase being more gradual on the alkaline than on the acid side. As the degree of alkalinity decreased from P_H 9·7 to 8·0 the increased precipitability decreased from 57·6 to 32·6 %; between P_H 8·0 and 5·5 it varied from 25 to 30 %, while between P_H 5·3 and 4·6 it rapidly rose from 40·0 to 75·5 %.

TABLE II. *Showing the influence of the hydrogen ion concentration on the denaturation of pseudoglobulin induced during the prolonged heating of a 7·95 % solution of pseudoglobulin at a temperature of 57·5° for 6 hours.*

Volume of normal acid or alkali added to 100 cc. of the pseudoglobulin solution	P_H of the solutions measured by the electrical method	Appearance of the heated liquids	*A* Percentage conversion of soluble into insoluble protein during the heating of the pseudoglobulin solutions	*B* Percentage increase in the precipitability of the pseudoglobulin by 30 % of saturation with ammonium sulphate
5·0 cc. *N* NH_3	9·72	clear	5·5	57·6
4·0	9·51	,, ...	5·5	57·0
3·0	9·40	,, ...	4·2	50·4
2·0	9·30	,, ...	1·3	44·0
1·50	9·03	very slightly opalescent ...	1·3	43·7
1·00	8·63	slightly opalescent	1·3	42·4
0·50	7·97	,, ,, ...	0	32·6
0·25	7·40	opalescent	0	29·2
0·00	6·81	very thin emulsion	0	25·6
0·50 cc. *N* HAc	6·43	thin emulsion, filtrate opalescent	1·3	26·3
1·00	5·99	thin emulsion, filtrate slightly opalescent	4·2	27·6
1·60	5·34	thick emulsion, filtrate clear	17·9	40·0
2·50	5·06	,, ,, ,,	43·0	48·2
3·30	4·80	,, ,, ,,	60·9	58·9
4·25	4·64	almost solid, filtrate clear ...	66·0	64·2
5·0	4·60	,, ,, ,, ...	78·0	75·5

C. THE EFFECT OF CHANGES IN [H·] ON THE HEAT DENATURATION OF ALBUMIN INDUCED DURING THE HEATING OF ITS SOLUTIONS AT 57·5° FOR A PERIOD OF SIX HOURS.

The solution of albumin was prepared as follows:

The filtrate from the precipitated globulins (ante, p. 295) was saturated with ammonium sulphate and the ensuing precipitate was filtered off. The precipitate was washed from the filter paper into three litres of a half-saturated solution of ammonium sulphate. The liquid after standing for twenty-four hours was filtered, and the filtrate was saturated with ammonium sulphate and allowed to stand for two days at room temperature. The precipitated albumin was filtered off, thoroughly drained, pressed and dialysed until the residue in the bags was practically free from sulphate. The residue from dialysis was diluted until the protein content was of the order of 6 % and 1·5 % of solid sodium chloride was then added.

To aliquot parts of the above solution of albumin were added varying amounts of acid and alkali so as to bring the [H·] of the series of experimental liquids within the ranges P_H 9·7 and 4·5; the [H·] of each of the solutions being measured by the electrical method previous to the liquids being heated at a temperature of 57·5° for a period of six hours.

The protein content of each of the above liquids and of the filtrates from

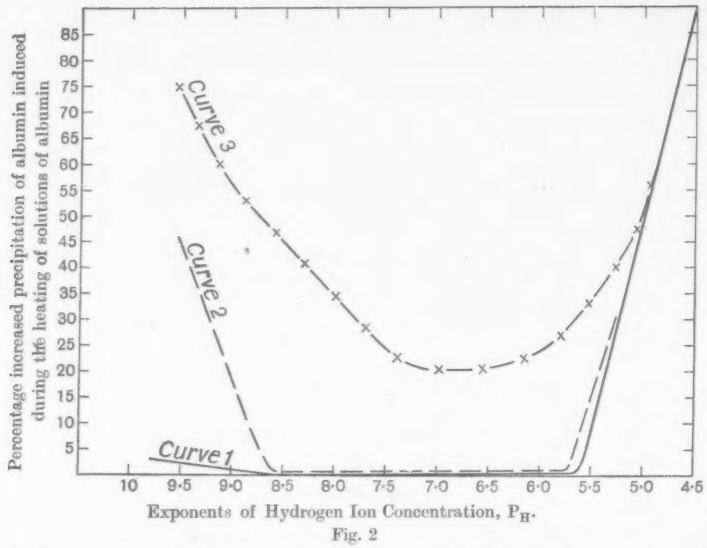

Fig. 2

Curve 1 ——— represents the influence of changes of reaction on the conversion of soluble albumin into an insoluble condition during the heating of solutions of albumin to 57·5° C. for 6 hours.

Curve 2 — — — represents the accompanying percentage increased precipitability of the heat denaturated albumin by 30 % of saturation with ammonium sulphate.

Curve 3 —×—×— represents the accompanying percentage increased precipitability of the heat denaturated albumin by 50 % of saturation with ammonium sulphate.

them made 30 % and also 50 % of saturation with ammonium sulphate were estimated before and after their having been heated.

The data thus obtained have been incorporated in Table III and have been plotted in Curves 1, 2 and 3 in Figure 2.

From a study of these data it will be seen that, as in the case of solutions of pseudoglobulin, the heated liquids on the alkaline side were either clear or else showed only a slight opalescence, whereas on the acid side the liquids were converted into thin suspensions the consistency of which became more markedly solid as the degree of acidity increased.

From the results given in Column *A* of Table III it will be seen that the actual conversion of soluble albumin into an insoluble condition was less than 5 % between the ranges P_H 9·5 and 5·5. On the acid side of P_H 5·5 it rapidly rose from 6·0 % at P_H 5·5 to 57·6 % at P_H 4·87 and to 100 % at P_H 4·40.

From the results given in Column *B* of the same table it will be seen that the precipitability of albumin by 30 % of saturation with ammonium sulphate is not appreciably affected during the heating of solutions of which the

TABLE III. *Showing the influence of the hydrogen ion concentration on the denaturation of albumin induced during the prolonged heating of a 5·20 % solution of albumin at a temperature of 57·5° for a period of 6 hours.*

Volume of normal acid or alkali added to 100 cc. of the solution of albumin	P_H of the solutions measured by the electrical method	Appearance of the heated liquids	Column *A* Percentage conversion of soluble into insoluble protein during the heating of the albumin solutions	Column *B* Percentage increase of the precipitability of the albumin by 30 % of saturation with ammonium sulphate	Column *C* Percentage increase of the precipitability of the albumin by 50 % of saturation with ammonium sulphate
4·3 cc. *N* NH_3	9·53	slightly cloudy	2·2	46·0	75·0
3·0	9·11	„ „	1·0	36·7	58·0
2·2	8·70	clear	0·0	8·0	50·0
1·00	8·41	„	0·0	0	44·0
0·50	7·81	„	0·3	0	31·5
0·25	7·40	„	0	0	22·7
0·00	6·90	„	0	0	21·3
0·70 cc. *N* HAc	6·27	slightly opalescent, filtrate opalescent	0	0	26·1
1·40	5·53	cloudy, filtrate opalescent ...	6·0	14·2	34·2
2·20	5·18	thin emulsion, filtrate clear ...	30·0	36·7	44·0
3·0	4·87	thick „ „ ...	57·6	55·0	57·0
6·0	4·52	„ „ „ ...	89·0	Too thick to be measured in a pipette	Too thick to be measured in a pipette
8·5	4·40	almost solid, filtrate clear ...	100		
6·0 cc. *N* H_2SO_4	3·40*	„ „ „ ...	100		

* Opalescent after the addition of acid.

reaction falls between the limits P_H 8·4 and 5·5. On either side of these limits there is a rapid rise in the precipitability of the albumin.

In Column *C* have been given the data with regard to the increased precipitability of the solution of albumin by 50 % of saturation with ammonium sulphate. It will be seen that the increased precipitability of the albumin is a function of the [H·] of the solutions. Thus, as the [H·] increased from P_H 9·5 to 7·8 so the increased precipitability decreased from 75 % to 31 %; between the values of P_H 7·8 and 5·5 it varied from 20 to 30 % while on the acid side of P_H 5·5 the percentage increased precipitability was practically equivalent to the percentage conversion of soluble into insoluble protein.

The results recorded above for pseudoglobulin and for albumin respectively show certain similarities with those previously obtained from corresponding experiments with antitoxic sera [Homer, 1917, 1]. Thus it was noticed that:

(1) The extent of the heat denaturation of the individual proteins, pseudoglobulin and albumin, is a function of the [H·] of the solutions.

(2) There is coincidence between the curves representing the conversion of soluble into insoluble protein during the heating of whole serum, of solutions of albumin and of pseudoglobulin respectively.

(3) The curve for the increased precipitability of albumin by 50 % of saturation with ammonium sulphate has a configuration very similar to those for the increased precipitability of pseudoglobulin and of the proteins of whole serum respectively by 30 % of saturation with ammonium sulphate.

(4) The values for the increased precipitability of the serum proteins by 30 % of saturation with ammonium sulphate are somewhat lower than those for solutions of pseudoglobulin at the same [H·]. This is probably due, partly to the diluent effect of the accompanying albumin in whole serum and partly to the "buffer" action of the latter.

(5) In all cases the minimum change takes place between the ranges P_H 5·5 and 8·0 and there is coincidence of the curves on the acid side of P_H 5·5.

(6) The changes taking place during the heating of alkaline sera and of alkaline solutions of pseudoglobulin and of albumin are not of the same type as those induced in the acid solutions, for acidification of the heated alkaline liquids to P_H 5·0 does not produce the thin suspension so characteristic of the heated acid liquids.

(7) The increased precipitability of the heated alkaline sera and of alkaline solutions of albumin and of pseudoglobulin only attains its maximum value after several hours' heating. On the other hand the changes in the acid liquids are practically complete within one hour's heating at the specified temperature.

From the work of Chick and Martin [1910, 1911, 1912, 1, 2] it was anticipated that while a certain similarity would exist between the configuration of the curves for the heat denaturation of pseudoglobulin and of albumin respectively at hydrogen ion concentrations on either side of their isoelectric points, there would be a shift in the position of corresponding portions of the curves due to the fact that the isoelectric point of pseudoglobulin is at P_H 5·52 while that of albumin is at P_H 4·70. Curiously enough not only was the region of least change the same in both cases, viz., from P_H 5·5 to 8·0, but there was absolute coincidence of the curves on the acid side of P_H 5·5.

These phenomena present points of interest in connexion with the views held both as to the justification for regarding the protein constituents of serum as well defined entities and as to the possible conversion of pseudo- into euglobulin and of albumin into pseudoglobulin.

The results of an investigation of the nature of the heat denaturated proteins obtained from albumin and pseudoglobulin will be dealt with in a later paper.

D. THE PRECIPITATION OF HEAT DENATURATED PSEUDOGLOBULIN AND HEAT DENATURATED ALBUMIN WITH THE FIRST AND SECOND FRACTION PRECIPITATES.

From a consideration of the above results it is obvious, that in sera which have been subjected to prolonged heating the First and Second Fraction precipitates respectively comprise the various proteins in proportions which are somewhat different from those in the corresponding precipitates in the unheated serum.

An investigation was therefore undertaken in order to furnish data with regard to the composition of the First and Second Fraction precipitates in heat denaturated sera throughout the ranges of acidity and alkalinity previously considered above in sections A, B and C.

The wider aspect of the problem has not yet been completed but, in view of the need for the immediate application of my results to routine concentration work, data are being published with regard to the precipitability of heat denaturated pseudoglobulin and albumin by ammonium sulphate from solutions the reaction of which had been adjusted to that of the routine batches of sera taken for concentration. The application of those results to the routine work has led to the production of end products in which the proportion of albumin has been lessened while the degree of concentration has been increased.

Solutions of pseudoglobulin and of albumin (prepared as in B and C), previous to their being heated to a temperature of 57·5° for six hours, were adjusted to about P_H 8·0. Samples of the heated and of the unheated liquids were made varying percentages of saturation with ammonium sulphate and were then filtered, the protein content of the filtrates being estimated by means of a Zeiss refractometer. From the data thus obtained was calculated the percentage conversion of pseudoglobulin and of albumin respectively from the emulsoid to the suspensoid condition induced by the addition of the various amounts of the electrolyte to the heated and to the unheated solutions.

The results obtained with pseudoglobulin have been embodied in Table IV. They show that in the unheated solutions the extent of the precipitation of pseudoglobulin increases with the concentration of ammonium sulphate, the change being complete at about 50 % of saturation with the electrolyte. In the heated solutions the precipitability of the protein by concentrations of ammonium sulphate beyond 20 % of saturation is considerably enhanced, the precipitation being practically complete at 42·5 % of saturation.

TABLE IV. *Showing the extent to which the precipitability of pseudoglobulin by ammonium sulphate is affected by the prolonged heating of its solution at 57·5° for 6 hours.*

[Percentage of pseudoglobulin in original solution = 7·10.]

P_H of the solution = 8·0.

Percentage of saturation with ammonium sulphate	Residual percentage of pseudoglobulin in solution in the filtrates from the ammonium-sulphate-pseudoglobulin mixtures		Percentage precipitation of pseudoglobulin by the ammonium sulphate in the	
	(*a*) before heating	(*b*) after heating	(*a*) unheated liquids	(*b*) heated liquids
20	6·92	6·77	2·5	4·6
25	6·77	5·65	4·6	20·5
28	6·67	4·56	6·0	35·9
30	6·04	3·77	15·0	47·0
33	3·86	2·76	45·5	61·2
37·5	1·65	0·75	79·5	89·5
42·0	1·14	0·19	84·0	97·5
44·5	0·54	0·03	92·5	99·5
47·5	0·26	0·00	96·5	100·0
50·0	0·07	0·00	99·0	100·0

In the case of albumin (Table V) similar results were obtained. Thus, in the unheated liquids at 44 % of saturation with ammonium sulphate about 6 % of the total albumin was converted from the emulsoid to the suspensoid state: at 50 % of saturation with the electrolyte the conversion was of the order of 10 %; beyond this point the precipitation of albumin gradually increased, the precipitation being complete at about 75 % of saturation. In the heated solutions the precipitation of albumin at concentrations of ammonium sulphate greater than 40 % of saturation was considerably increased. Thus, at 42 % of saturation the precipitation was of the order of 29 % of the total albumin; at 44 % of saturation it increased to 33, while at 50 % of saturation the precipitation of albumin rose to the order of 52 %.

E. THE PRACTICAL APPLICATION OF THE ABOVE RESULTS TO THE ROUTINE CONCENTRATION OF ANTITOXIC SERA.

While recognising that the values recorded in Tables IV and V for the percentage precipitation of the individual proteins from their respective solutions may be somewhat higher than when they are associated together in serum, it seemed justifiable to conclude that, in the routine work, by lowering the concentration of ammonium sulphate for the precipitation of the Second Fraction from 50 to 44 % of saturation, there would still be complete precipitation of the pseudoglobulin and antitoxin while the proportion of albumin precipitated at this stage would be appreciably lessened.

TABLE V. *Showing the extent to which the precipitability of albumin by ammonium sulphate is affected by the prolonged heating of its solution at 57·5° for 6 hours.*

[Percentage of albumin in the original solution = 6·27.]

P_H of the solution = 8·24.

Percentage of saturation with ammonium sulphate	Residual percentage of albumin in solution in the filtrates from the ammonium-sulphate-albumin mixtures		Percentage precipitation of albumin by the ammonium sulphate in the	
	(*a*) before heating	(*b*) after heating	(*a*) unheated liquids	(*b*) heated liquids
20	6·27	6·27	0	0
25	6·27	6·27	0	0
28	6·05	6·14	3	2
30	6·14	6·02	2	4
33	6·05	5·83	3	7·1
37·5	5·95	5·61	5	10·6
42	5·92	4·46	5·5	29·0
44·5	5·84	4·20	6·7	32·9
47·5	5·77	3·86	8·0	38·4
50·0	5·64	2·98	10·0	52·5
56·5	4·76	1·10	23·7	82·5
62·5	3·45	0·81	45·0	87·0
66·5	0·73	0·15	88·5	97·5
70·0	0·18	0·00	97·0	100·0

In order to test this point concentrations were carried out with antidiphtheritic and with antitetanic sera. In both cases a denaturation of the serum proteins to the extent of about 30 % was induced during the initial heating of the serum. Aliquot parts of the filtrates from the First Fraction precipitates were respectively brought up to 44, 47 and 50 % of saturation with ammonium sulphate and the ensuing precipitates were filtered, pressed and dialysed in the usual way.

TABLE VI. *Showing the increase in the degree of concentration of antitoxic sera induced by lowering the precipitation limit for the Second Fraction precipitate.*

Precipitation limits for the Second Fraction precipitates (percentage of saturation with ammonium sulphate)	Percentage of the total antitoxic units appearing in the Second Fraction precipitates	Percentage removal of the total proteins of the original serum	Increased potency per cc.	Units of antitoxin per g. of protein in the final product
Antitetanic plasma*				
30–44	95	69	10 times	6,250
30–50	95	64	8 „	5,250
Antidiphtheritic plasma*				
30–44	92	72·5	9·5 „	18,750
30–47	96	67	8·0 „	16,500
30–50	96	66	7·5 „	15,000

* Comparable results were obtained irrespective of whether the heat denaturation had been induced by the prolonged heating of serum at 57·5° or by the shortened heating process after the addition of cresylic acid to the serum.

A comparison was then made between the removal of the serum proteins, the increased potency and the relative recovery of antitoxin in the several experiments (Table VI).

From these results it is obvious that for the complete precipitation of the antitoxin and the pseudoglobulin from sera in which a heat denaturation of about 30 % has been induced, the concentration of ammonium sulphate need not be greater than 44 % of saturation.

The adoption of 44 % of saturation with ammonium sulphate in preference to 50 % hitherto recommended presents the following advantages:

(1) The precipitation of heat denaturated albumin with the Second Fraction precipitate is appreciably lessened, thereby ensuring in the end product:

(*a*) a further reduction in the percentage of the total proteins of the original serum without transference of antitoxin to the First Fraction precipitate, and

(*b*) a corresponding increase in the degree of concentration.

(2) The colour of the end products is much less pronounced than when the precipitation limit of 50 % of saturation is used. The extra amount of albumin precipitated between 44 and 50 % of saturation adsorbs an appreciable amount of the colouring matter of serum.

(3) The smaller volume of saturated solution of ammonium sulphate thus required for the precipitation of the Second Fraction precipitate entails a slight reduction of labour and expense.

From the above observations it is clear that, if the precipitation limits for the Second Fraction precipitate be altered so as to exclude the precipitation of heat denaturated albumin, the maximum load of antitoxin which can be carried by one gram of protein in the final product will be somewhat higher than the values previously found [Homer, 1917, 2]. Experimental work in this connexion is in progress and will be published later.

Summary.

During the course of the investigation it has been shown that:

1. The heat denaturation of the serum proteins in alkaline sera is a function of the duration of the time of heating. The maximum effect is produced after four hours' heating at 57·5°.

2. In acid sera the change is complete within one hour's heating at the specified temperature.

3. The heat denaturation of pseudoglobulin and of albumin is a function of the [H·] of their solutions.

4. There is a marked similarity between the curves for the conversion of soluble into insoluble protein during the heating of serum, of solutions of albumin, and of pseudoglobulin throughout the ranges P_H 4·6 to 8·0.

5. The curves for the increased precipitability of the serum proteins and of pseudoglobulin by 30 % of saturation with ammonium sulphate and for the increased precipitability of albumin by 50 % of saturation with ammonium sulphate show a minimum amount of change through the region P_H 5·5 to 8·0 while on the acid side of P_H 5·5 there is absolute coincidence of the curves.

6. In heat denaturated sera the protein precipitated between 30 and 50 % of saturation with ammonium sulphate contains a mixture of pseudoglobulin, antitoxin and heat denaturated albumin.

7. The precipitation of pseudoglobulin from solutions of which the reaction, previous to the liquids being heated, had been adjusted approximately to P_H 8·0 was complete between 42 and 44 % of saturation with ammonium sulphate.

8. The precipitation of albumin from solutions of which the reaction, previous to the liquids being heated, had been adjusted approximately to P_H 8·0 gradually rose from 29 to 52 % between 42 and 50 % of saturation with ammonium sulphate.

9. The practical application of the above observations to the routine concentration of sera has shown us that:

(*a*) The preliminary prolonged heating of sera of which the reaction is on the alkaline side of P_H 7·0 must be for not less than 4 hours and not more than 5 hours at 57·5°.

(*b*) In methods involving a heat denaturation of the serum proteins, the amount of heat denaturated albumin appearing in the final product can be appreciably lessened by including in the Second Fraction precipitate the protein precipitated between 30 and 44 % of saturation with ammonium sulphate instead of between 30 and 50 % of saturation as hitherto employed.

(*c*) This decreased precipitation of albumin entails the production of end products showing a less pronounced colour, a greater removal of the total proteins of the original serum and a degree of concentration considerably greater than if the higher precipitation limit had been used.

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XXVIII. THE FORMATION OF CREATINE. EFFECTS ON THE EXCRETION OF CREATINE IN THE BIRD PRODUCED BY PARAFORMALDEHYDE AND HEXAMETHYLENE-TETRAMINE GIVEN SEPARATELY AND COMBINED WITH ARGININE CARBONATE AND OTHER SUBSTANCES.

By WILLIAM HENRY THOMPSON.

(With an Addendum by Emil Alphonse Werner.)

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In a series of investigations extending over the past five years, the results of which have been recently published [Thompson, 1917, 1], it has been shown that when arginine is administered to animals a portion of its guanidine nucleus is methylated and either excreted in the urine, or stored in the muscles as creatine, or both. The proportion methylated was greater when the substance was given by hypodermic injection than when added to the food. It was also increased by combining with the arginine (particularly if hypodermically injected) substances containing a methyl group such as methyl citrate and methyl benzoate [Thompson, 1917, 2].

When given with food to dogs on non-meat diet, 2·5 % of methylated guanidine nucleus appeared in the urine: in the case of dogs on meat diet 14·6 %. This latter figure was however admittedly open to question owing to the difficulty of ensuring a constant amount of creatine in the food.

If given hypodermically, dogs on non-meat diet excreted 4·5 % of the guanidine nucleus in the urine, while one dog on a meat diet excreted 4·58 %.

In birds the output of methylated guanidine nucleus when arginine was given with the food, amounted to 1·1 %, if given by hypodermic injection to 2·5 %, of that administered.

Experiments to test the effect of intravenous injection of arginine on the creatine content of rabbit's muscle, showed that there was invariably an increase, which corresponded on the average to a storage in the muscles of

14·5 % of the methylated guanidine nucleus, the variations lying between 8 % and 25 %. At the same time there was an output of the methylated nucleus in the urine secreted in the first three hours after the injection amounting to 0·1 % of the quantity injected.

For the dog the average percentage partition of arginine nitrogen in the urine (*a*) when given with food, (*b*) when given by hypodermic injection was as follows:

	Total nitrogen recovered	Urea nitrogen	NH_3 nitrogen	Amino-acid N	N as total creatinine	N not recovered in the period
(*a*) Given with food (5 expts.)	56·5 %	34·7 %	13·7 %	2·33 %	3·47 %	43·5 %
(*b*) Injected	67·87	35·4	4·05	4·7	4·12	32·13

These results amplify and confirm those of previous experiments [Thompson, 1905, 1906].

The main conclusion indicated by these facts is that when a complex substance such as arginine is introduced into the animal body—more especially after reaching the tissues—it is attacked in various ways and dealt with by different agencies.

Many attempts were made to influence the degree of methylation of the guanidine nucleus but in the majority of cases with little obvious effect, so far as the excretion of total creatinine in the urine could be taken as an index. Various circumstances however pointed to the possibility that the presence of formaldehyde in the tissues and the extent to which it is produced, affect the results. Formaldehyde has long been known to be produced by the tissues in plant metabolism. It is also probable that this accounts for the formation of methylated compounds such as betaïne, etc., in many vegetables. Betaïne has also been shown to be present in the muscle of invertebrate animals whereas creatine is absent. This has been established by Kutscher [1914] for the crab and by D. Wright Wilson [1914, 1, 2] for the scallop and periwinkle. On passing to a stage higher in the animal scale, namely to the lamprey, the latter author was able to isolate both betaïne and creatine from the extracts of its muscle. Arginine was also found by these observers to be present in invertebrate muscle. The tissues of invertebrates thus resemble those of plants in the absence of creatine and the presence of betaïne. Further the tissues of lower animals contrast with those of the higher in the presence of betaïne and the predominance of simple amino-acids in the former, whereas in the latter there is usually an excess of diamino-acids and complex methylated basic extractives such as creatine, carnitine, oblitine, etc.

It seemed therefore probable that formaldehyde, which appears undoubtedly to be produced in the tissues of invertebrates is also formed to some extent in those of higher animals. The presence of formic acid as an excretory product in the urine also lends support to this hypothesis.

Reasoning from these considerations and assuming that under normal conditions the production of formaldehyde in the tissues of higher animals is small, it was inferred that the introduction of formaldehyde combined with arginine would probably result in an increased degree of methylation of its guanidine nucleus, as shown by an increased formation and excretion of creatine.

Preliminary observations were made on two birds to test this hypothesis by noting the effects on the excretion of creatine in the urine. It did not seem advisable however to use formaldehyde as such, owing to its violent action on tissues, either for feeding or hypodermic injection. At the suggestion of my friend Dr Emil Werner, Professor of Applied Chemistry in Trinity College, Dublin (whom I consulted on the possible chemical reactions involved) paraformaldehyde was used instead. This in solution is slowly converted into formaldehyde, and proved very suitable for the experiments. The following is a summary of the results.

TABLE I. *The effects on the excretion of creatine of paraformaldehyde and arginine given with food and injected.*

Animal used	No. of days and quantity given	Creatinine (total) excreted per day in mg. (a) before	(b) during	(c) after	Effect on weight of animal in g.
Duck 8	2 days, arg. 1 g., paraformald. 0·3 g., with food	6·72	15·09	8·21	+ 7
„ 8	1 day, same quantities, with food	8·21	8·00	8·16	+40
„ 9	2 days, same	21·61	34·12	31·10	+20
„ 9	1 day, same quantities, injected	8·99	50·13 1st day 70·92 2nd „ 21·90 3rd „	8·27	+16

Only in two of the observations in which paraformaldehyde was given with food were effects seen on the excretion of total creatinine in the urine. It is to be remarked however that the third case was a "repeat" observation and in such cases as previously pointed out there is usually a decline or actual reversal of the effect on the excretion in the urine. In the two cases where an effect was seen, the increase of total creatinine corresponded to a methylation of 2·2 % of the guanidine nucleus given, being double the amount

observed as stated in previous experiments with birds when arginine alone was given with the food.

Turning now to the experiment in which paraformaldehyde and arginine were given by subcutaneous injection it will be seen that the effects were very pronounced, much more so than in any of the previous experiments with birds. Only one injection was made, the effects of which lasted for three days. During this period there was an average daily excretion of 47·65 mg. as compared with the normal daily average of 8·99 mg. That is to say in the three days there was an excess of total creatinine in the urine amounting to 115·98 mg. over the normal, which represents an excretion in methylated condition of 24·2 % of the guanidine nucleus given. It may be recalled that the injection of arginine alone in previous experiments led to a corresponding excretion of 2·5 % of its guanidine nucleus. It cannot however be taken as definitely proven that the whole of the methylated guanidine nucleus excreted in the recent experiments in excess of the normal, was actually derived from the arginine injected. The results given in the following table seem to indicate the contrary since the injection of paraformaldehyde alone was followed also by a great increase in the excretion of total creatinine.

TABLE II. *The effects on the excretion of creatine of paraformaldehyde alone, given with the food and injected.*

Animal used	No. of days and quantity given	Creatinine (total) excreted per day in mg. (a) before	(b) during	(c) after	Effect on weight of animal in g.
Duck 8	2 days, 0·3 g., with food	8·03	9·92	6·72	+25
„ 9	2 days, same	13·74	12·55	15·75	−50
„ 8	1 day, 0·3 g., injected	11·79	79·36 1st day 63·42 2nd „	10·31	nil

It will be seen that in the two observations in which paraformaldehyde was given with the food there was no appreciable effect produced on the excretion of creatinine, while in the third in which the substance was injected there was a marked increase which lasted for two days. During this period the excess excretion of total creatinine over the normal, amounted to 109·2 mg. This excess cannot however be strictly compared with the corresponding excess seen after the injection of paraformaldehyde and arginine combined, since the two observations were made on different birds. Further experiments are needed to settle beyond doubt whether or not the simultaneous injection of arginine with paraformaldehyde produces a decidedly greater effect on output of total creatinine than the latter alone.

One thing however seems unquestionably to be established, namely that formaldehyde is in part at all events disposed of in the tissues by combination with a guanidine nucleus to form creatine. This is confirmed also by results obtained from the injection of urotropine (hexamethylene-tetramine), a substance which is known to liberate formaldehyde in the tissues. Thus

$$N_4(CH_2)_6 + 6H_2O = 6CH_2O + 4NH_3.$$

Three observations were made on the same bird, one in which urotropine and arginine were given with the food, a second in which urotropine alone was similarly given, and a third in which the last-named substance was given by subcutaneous injection. The results are shown in Table III. On examining them it will be seen that in the observations in which the substances were added to the food, urotropine alone produced no appreciable effect; whereas when arginine was given along with it the output of creatinine was more than doubled. When the same quantity of urotropine was *injected* but without arginine, the effect which followed lasted three days and even then had not

TABLE III. *The effects on the excretion of creatine of urotropine (hexamethylene-tetramine) and arginine given with food, and of urotropine alone given with food and injected.*

Animal used	No. of days and quantities given	Creatinine (total) excreted per day in mg. (a) before	(b) during	(c) after	Effect on weight of animal in g.
Duck 9	1 day, urotropine 0·3 g., with food	8·27	10·07	—	− 5
„ 9	1 day, arg. 1 g., urotropine 0·3 g., with food	8·27	18·99	9·83	+10
„ 9	1 day, urotropine 0·3 g., injected ...	9·83	13·11 1st day 24·13 2nd „ 20·68 3rd „	12·27	−20

fully subsided. During this period an excess of 28·43 mg. of creatine over and above the normal (9·83 mg. per day) was excreted. That is to say on the average the output was doubled for three days following a single injection. If the whole of the formaldehyde theoretically liberated from the quantity of hexamethylene-tetramine injected were disposed of by combination with a guanidine nucleus and forthwith excreted, the output of creatine should have been much greater, that is approximately equal to the output after a corresponding injection of paraformaldehyde. Further experiments are needed before an explanation of the difference in the two cases can be assigned.

Three observations were also made on the effects of combining paraformaldehyde with substances other than arginine. The substances used

were glycine, sarcosine and guanidine carbonate, and in all cases they were given with the food. The results are shown in Table IV. On examining them it will be seen that in all cases there was an increased output of total creatinine during the feeding period. The effects are small in comparison with some of those considered in this paper but by no means negligible, the increase in each case being approximately 60 % over the normal.

TABLE IV. *The effects on the excretion of creatine of paraformaldehyde given in the food with other substances than arginine.*

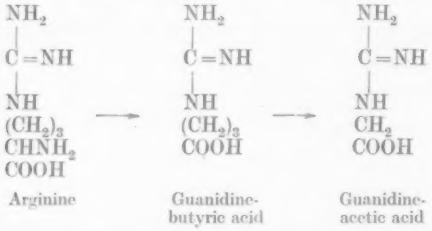
Animal used	No. of days and quantities given	Creatinine (total) excreted per day in mg. (a) before	(b) during	(c) after	Effect on weight of animal in g.
Duck 8	2 days, glycine 1 g., paraformald. 0·3 g. ...	6·94	11·40	—	+20
,, 8	1 day, sarcosine 1·3 g., paraformald. 0·3 g....	6·94	11·79	—	+30
,, 8	2 days, guanidine carb. 0·25 and 0·3 g., paraformald. 0·25 g.	4·67	7·06	6·94	−20

Observations were also made in each case on the output of preformed creatinine but in no case was the effect appreciable.

DISCUSSION.

It remains to suggest a chemical hypothesis by which the foregoing facts might be explained.

It has been pointed out by Neubauer [1910], Dakin [1912, p. 62] and others that in accordance with typical oxidation processes known to occur in the animal body guanidine-acetic acid (glycocyamine) can readily be derived from arginine, the stages being as follows:



Arginine — Guanidine-butyric acid — Guanidine-acetic acid

It has also been conclusively proved [Jaffé, 1906; Dorner, 1907; Thompson, 1917, 1] that the last-named body is methylated in the tissues with the production of creatine, which is partly stored in the muscles and partly excreted in the urine.

No suggestion has been offered as to how this methylation occurs, but it is not improbable that in the production of creatine from guanidine-acetic acid the methylation arises from the action of formaldehyde. The reaction may provisionally be represented in the following way:

(1) $HN:C\begin{cases}NH_2\\NHCH_2COOH\end{cases} + CH_2O = HN:C\begin{cases}N:CH_2\\NHCH_2COOH\end{cases} + H_2O$

(2) $HN:C\begin{cases}N:CH_2\\NHCH_2COOH\end{cases} + CH_2O + H_2O = HN:C\begin{cases}NHCH_3\\NHCH_2COOH\end{cases} + HCOOH$

By migration of the CH_3 group which is more likely to occur in the animal body than in vitro, the isomer of creatine,

$$HN:C\begin{cases}NHCH_3\\NHCH_2COOH\end{cases}$$

would become

$$HN:C\begin{cases}NH_2\\N(CH_3)\,CH_2COOH\end{cases}$$

This hypothesis has the merit that the results obtained from the injection of arginine and paraformaldehyde both singly and combined could be explained on similar lines. It is improbable however that the methylation of the arginine would be deferred till, by the process of oxidation, it had reached the stage of glycocyamine. It is more likely that methylation and oxidation go hand in hand, the end results being the production of creatine, ammonia, carbon dioxide and water, the major part of the NH_3 and CO_2 appearing in the urine as urea.

Before considering this suggestion further it is necessary to refer to Sörensen's results [1908] on the interaction of formaldehyde and amino-compounds. With neither arginine (free base) nor with guanidine was acidity immediately developed when titration with neutral formaldehyde was carried out. Acidity was however obtained when he used arginine hydrochloride. Sörensen concluded from these results that the amino-group of the arginine reacts as usual with formaldehyde but that the guanidine group on the contrary is unable to fix the aldehyde.

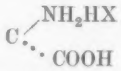
I am indebted to my colleague, Prof. E. Werner, for a critical discussion of Sörensen's results and conclusions in which he states that the results are exactly what he would have predicted but that the inferences are not what he would have drawn. Nor would the acidity in the case of arginine hydrochloride be entirely due to a liberation of HCl but to the production also of formic acid. Werner holds, and I think rightly, that when CH_2O is added

to any compound containing NH_2 in such a complex as occurs in all the amino-acids the result may be expressed in general terms as follows:

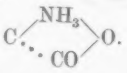
(1) $C...NH_2 + CH_2O = C...N : CH_2 + H_2O$,

(2) $C...N : CH_2 + CH_2O + H_2O = C...NH . CH_3 + HCOOH$.

In the case of guanidine a methylguanidine[1] is formed which neutralises the formic acid as fast as it is produced, hence the neutrality observed by Sörensen. In the case of arginine free base the guanido-group is methylated and neutral methylated arginine formate is produced. If the arginine be present as a stable salt, this salt is formed by the acid (say HCl) being attached to the guanido-group



while on the "cyclic" or "internal ammonium salt" theory of the structure of amino-acids there would be mutual neutralisation of the NH_2 and COOH end groups as here indicated:



Hence the formic acid produced would remain free, the guanido-group being neutralised by the HCl. This explanation implies that in all cases where acidity is developed by titrating a neutral amino-acid compound with neutral formaldehyde, formic acid is set free, at all events, to some extent. (See Addendum, p. 318.)

Applying these considerations to the interaction of arginine and formaldehyde in the tissues the stages would conceivably be those already suggested (p. 312).

The arginine would thus by the processes of methylation and oxidation pass through the stages of (1) methylene-guanido-butyric acid, (2) methylene-guanido-acetic acid, (3) to an isomer of creatine which by migration of the methyl group becomes ordinary creatine.

The scheme at all events provides a working hypothesis, which will I believe, in the main if not in all its details, prove to be correct. I had hoped to find time before publishing this preliminary account to test on the animal two of the points which could readily be confirmed or the reverse, namely (1) whether the combination of formaldehyde with glycocyamine would increase the methylation of the latter, (2) whether formic acid appears or is increased in the urine as a result of the injections. Unfortunately, however, although I have

[1] A methylene-guanidine formed in accordance with equation (1) would no doubt be itself a strong base and hence no acidity would be developed. In a lesser degree this would also hold for arginine (free base).

waited a year in the hope of finding the necessary time this has not proved possible and the prospect is so distant that I am constrained to publish the investigation in its present incomplete condition. Obviously much has yet to be done for which it is hoped that opportunity will ultimately present itself.

In conclusion I have to express my indebtedness to Dr Werner for his kind criticism and valuable suggestions.

Summary.

1. The administration of arginine and paraformaldehyde with food to ducks was followed by an increase in the excretion of creatine which corresponded to a methylation of 2·2 % of the guanidine nucleus contained in the arginine. Previous experiments with arginine alone, given in the same way led to a corresponding excretion of 1·1 % of the guanidine nucleus. Paraformaldehyde alone produced no effect.

2. Arginine and paraformaldehyde given to the same birds by subcutaneous injection led to an excess excretion of creatine which represented a methylation of 24·2 % of the guanidine nucleus of the arginine. In previous experiments with arginine alone the corresponding amount methylated and excreted was 2·5 % of the guanidine nucleus. Paraformaldehyde injected alone also caused a large excess in the excretion of creatine. It is concluded therefore that formaldehyde is disposed of in animal tissues, in part at least, by combination with a guanidine nucleus to form creatine.

3. Arginine and hexamethylene-tetramine similarly given with food to one of the ducks increased the output of creatine in the urine to double the normal. Hexamethylene-tetramine alone had no effect.

4. Hexamethylene-tetramine given by subcutaneous injection to the same bird, doubled the output of creatine for three days after a single injection. The whole of the formaldehyde theoretically obtainable and presumably liberated in the tissues from the quantity of hexamethylene-tetramine used, cannot have been disposed of by combination with a guanidine nucleus or if so, the creatine formed was not forthwith excreted in the urine.

5. Paraformaldehyde given in the food along with glycine, sarcosine, and guanidine carbonate respectively increased the excretion of creatine in the urine by about 60 % in each case.

6. No appreciable effect was produced by any of the substances on the excretion of preformed creatinine.

7. A chemical explanation is offered to account for the rôle played by formaldehyde in the methylation of the part of the guanidine nucleus of arginine converted into creatine in the animal body.

PROTOCOLS.

Duck VIII. In each case the days marked with letters are those on which the effects appeared, that is to say, the administration of the substance was begun a day earlier than that marked. Lines of space across the page are introduced to mark off the periods.

Date 1916	Preformed creatinine per day in mg.	Total creatinine per day in mg.	Total nitrogen per day in mg.	Weight kilos
June 16	4·24	8·17	0·698	1·495
„ 17	4·11	5·85	0·669	1·495
„ 18	4·16	10·06	0·734	1·540
„ 19 (*a*)	4·17	**12·47**	0·718	1·560
„ 20 (*a*)	2·81	**7·37**	0·561	1·565
„ 21	2·41	7·31	0·581	1·540
„ 22	2·01	6·14	0·664	1·550
„ 23 (*b*)	2·91	**15·33**	0·485	1·560
„ 24 (*b*)	2·66	**14·87**	0·595	1·545
„ 25	3·85	9·14	0·598	1·520
„ 26	2·82	7·28	0·660	1·540
„ 27 (*c*)	2·62	**8·00**	0·559	1·580
„ 28	3·36	8·16	0·428	1·560
„ 29	2·73	4·67	0·625	1·565
„ 30 (*d*)	3·13	**7·77**	0·556	1·550
July 1 (*d*)	2·79	**6·35**	0·599	1·545
„ 2	2·42	6·94	0·609	1·580
„ 3 (*e*)	4·33	**10·82**	0·819	1·590
„ 4 (*e*)	3·98	**11·98**	0·859	1·600
„ 5 (*f*)	4·06	**11·79**	0·777	1·630
„ 6 (*g*)	4·01	**79·36**	0·985	1·630
„ 7	4·06	**63·42**	0·708	1·630
„ 8	3·71	13·12	0·736	1·625
„ 9	3·66	7·50	0·681	1·630

(*a*) Paraformaldehyde 0·3 g. with food.
(*b*) Paraformaldehyde 0·3 g., arginine carb. 1 g. with food.
(*c*) Paraformaldehyde 0·5 g., arginine carb. 1 g. with food.
(*d*) Paraformaldehyde and guanidine carbonate with food, first day 0·25 g. of each, second day 0·25 g. of paraformaldehyde and 0·3 g. guanidine carbonate.
(*e*) Paraformaldehyde 0·3 g., glycine 1 g. with food.
(*f*) Paraformaldehyde 0·3 g., sarcosine 1·3 g. with food.
(*g*) Paraformaldehyde 0·3 g., injected.

Duck IX.

Date 1916	Preformed creatinine per day in mg.	Total creatinine per day in mg.	Total nitrogen per day in g.	Weight kilos
June 10	2·53	13·33	0·387	1·770
„ 11	3·14	14·16	0·386	1·750
„ 12 (*a*)	3·57	12·35	0·422	1·770
„ 13 (*a*)	3·49	12·74	0·444	1·740
„ 14	2·97	13·16	0·374	1·720
„ 15	4·03	17·38	0·355	1·725
„ 16	4·08	18·75	0·402	1·725
„ 17	4·25	20·83	0·315	1·720
„ 18	3·82	22·39	0·300	1·700
„ 19 (*b*)	3·83	**28·43**	0·567	1·710
„ 20 (*b*)	2·71	**39·80**	0·453	1·720
„ 21	4·49	**40·78**	0·517	1·690
„ 22	4·44	**21·43**	0·471	1·720
„ 23	2·30	7·78	0·429	1·730
„ 24	3·61	8·05	0·297	1·725
„ 25	3·37	9·93	0·318	1·705
„ 26 (*c*)	4·16	**50·13**	0·644	1·750
„ 27	3·75	**70·92**	0·506	1·725
„ 28	3·25	**21·90**	0·446	1·740
„ 29	3·26	8·11	0·402	1·750
„ 30	3·97	8·43	0·346	1·760
July 1 (*d*)	3·45	10·58	0·475	1·750
„ 2 (*d*)	4·19	9·55	0·470	1·750
„ 3 (*e*)	5·70	**15·69**	0·678	1·700
„ 4 (*e*)	4·39	**22·30**	0·625	1·760
„ 5	3·76	9·83	0·315	1·750
„ 6 (*f*)	5·13	**13·11**	0·567	1·730
„ 7	4·69	**24·13**	0·645	1·700
„ 8	4·12	**20·68**	0·465	1·710
„ 9	—	12·06	0·449	1·710
„ 10	4·29	12·48	0·649	1·710

(*a*) Paraformaldehyde 0·3 g. with food.
(*b*) Paraformaldehyde 0·3 g., arginine carb. 1 g. with food.
(*c*) Paraformaldehyde 0·3 g., arginine carb. 1 g. injected.
(*d*) Hexamethylene-tetramine 0·3 g. with food.
(*e*) Hexamethylene-tetramine 0·3 g., arginine carb. 1 g. with food.
(*f*) Hexamethylene-tetramine 0·3 g. injected.

ADDENDUM.

By Emil Alphonse Werner.

It may be well to point out the following facts, in connexion with equations (1) and (2) given by Prof. Thompson (p. 313), which briefly represent the mechanism of the methylation of amino-compounds by formaldehyde in accordance with the theory recently put forward by the writer [Werner, 1917].

Whilst equation (1) expresses the main change upon which Sörensen's titration method is based; three conditions are necessary for the promotion and completion of equation (2). These are, (*a*) rise of temperature, (*b*) the presence of formaldehyde in excess, and (*c*) the production of a methylene-amino-compound of the type $:C.N:CH_2$, more or less prone to reduction. It is obvious that in Sörensen's method, condition (*b*) is always fulfilled, whilst (*c*) will depend upon the nature of the amino-compound under investigation.

It will be seen therefore, that it is not suggested by the theory in question, that equation (2) is completed under the conditions employed by Sörensen, but that it may proceed simultaneously with equation (1) in certain cases seems highly probable.

So far as the interaction of formaldehyde and arginine with the ultimate formation of creatine is concerned, it would appear from a purely chemical point of view, that the scheme put forward by Prof. Thompson gives a very plausible explanation of the series of changes.

It may be fairly assumed that in an animal body the conditions would be favourable for the promotion of equation (2).

In conclusion, it may be pointed out that the velocity of reaction (2) is enhanced by another condition, namely the removal of formic acid as it is produced, either by neutralisation, or by its oxidation to carbon dioxide, and water.

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XXIX. THE PREPARATION OF PLANT NUCLEIC ACIDS.

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(*Received August 10th*, 1917.)

The methods described in the literature for the preparation of plant nucleic acids involve the extraction and separation of nucleoproteins containing varying amounts of phosphorus, and subsequent digestion with pepsin hydrochloric acid mixture until a product giving no biuret reaction and containing 8–9 % phosphorus is obtained. Several fermentations and treatment with picric acid are required to obtain a biuret-free substance. The methods are somewhat cumbersome and necessitate the manipulation of large volumes of liquid.

Nucleic acid of plant origin was required in some quantity for biological experiments and the following work was undertaken with the object of finding out if its preparation could be simplified and a biuret-free product obtained without peptic digestion.

Preliminary experiments were made with the raw material used—wheat embryos and dried yeast—by quantitative extraction with water and with solutions of sodium acetate or sodium chloride and afterwards with very dilute caustic potash. The proportion of the total phosphorus extracted by the various solutions was thus ascertained and a rough indication obtained of the form in which this element existed.

The results are given below.

Wheat embryo (1·13 % *total phosphorus*).

Percentage of the total phosphorus extracted by	10 % sodium acetate	= 63·7
,, ,, ,, ,,	*N*/10 caustic potash	= 32·5
	Total	= 96·2
Percentage of the total phosphorus extracted by	10 % sodium chloride	= 85·2
,, ,, ,, ,,	*N*/20 caustic potash	= 12·4
	Total	= 97·6
,, ,, ,, ,,	Water	= 75·7

Wheat embryo (another sample) (0·78 % *total phosphorus*).

Percentage of the total phosphorus extracted by 10 % sodium chloride = 80·4

Dried, alcohol-extracted yeast (2·33 % *total phosphorus*).

Percentage of the total phosphorus extracted by 10 % sodium chloride = 57·2

In the above experiments the extraction was carried out at a temperature 60–80°, and in cases where a second extraction of the same material was made the residue was thoroughly washed with water on the centrifuge.

A very large proportion of the total phosphorus of wheat embryos is soluble in a 10 % solution of sodium chloride and if this extraction is followed by extraction with very dilute alkali, practically the whole of the phosphorus is removed. Some of the phosphorus thus extracted is combined in the form of nucleic acid and can be separated by the addition of hydrochloric acid to the NaCl solution, the nucleic acid being combined as a salt with varying amounts of protein.

The remainder of the phosphorus is present as phytin ($C_{12}H_{22}O_{44}P_{10}Ca_7Mg$) and can be isolated in this form after the separation of nucleic acid by the methods already described by one of the authors [Clarke, 1914].

From dried yeast less of the phosphorus is extracted with sodium chloride solution, although a somewhat larger yield of nucleic acid is obtained from this material than from wheat embryos. A considerable proportion of the phosphoric acid appears to exist in dried yeast in an uncombined form.

As pointed out by Osborne and Harris, nucleic acid exists in plant tissues combined with varying amounts of proteins in the form of salts, the number of which known to exist in plants is exceedingly large. These salts, which contain in many cases only a small amount of phosphorus, retain their protein characters and the phosphorus was formerly regarded as part of the protein molecule.

During the course of the present investigation in which untreated raw material was extracted with 10 % sodium chloride solution and the clear extract treated with hydrochloric acid, substances were obtained in which the phosphorus varied from an inconsiderable trace to 3 %, and which all contained large amounts of protein and gave a well defined biuret reaction.

The problem of obtaining protein-free nucleic acid by direct extraction therefore, resolved itself into devising some means of so altering the protein complex that it was no longer extracted with sodium chloride solution. It was found that boiling the material used with a large excess of 95 % ethyl

alcohol for two hours and subsequently extracting the dried material with warm 10 % sodium chloride solution gave a clear extract from which by the addition of hydrochloric acid a characteristic precipitate of nucleic acid could be obtained that quickly settled and could be ground to a fine powder. This substance gave no biuret reaction and contained in the case of yeast 7·79 % phosphorus, and in the case of wheat embryo 4·6 % phosphorus. The wheat embryo employed was mixed with a considerable amount of endosperm and the crude nucleic acid obtained from this source was mixed with starch. This could be removed by suitable treatment during the subsequent purification but the presence of gelatinised starch in the extract was very inconvenient and caused a loss of a certain amount of nucleic acid.

In later preparations from wheat embryo the starch was gelatinised by placing the alcohol-extracted material in boiling water and heating. The starch was then fermented with taka-diastase in the presence of toluene until the solution no longer gave the starch reaction with iodine. Salt was added in sufficient quantity to give a 10 % solution and the extraction and separation of the nucleic acid continued in the usual way. This method gives extracts that can be easily filtered and a starch-free product.

Preparation of nucleic acid from yeast.

30 lbs. of freshly pressed yeast were treated with a large excess of 95 % alcohol, allowed to stand 24 hours, filtered on a Buchner funnel and dried in air. The air-dried material, in successive batches of 1000 g. was boiled for two hours with 95 % alcohol, filtered, pressed and dried in a current of air at 37°. It was then ground to a fine powder in a laboratory mill. 1000 g. of finely ground material, prepared in the above manner was extracted with 10 litres of 10 % sodium chloride at a temperature 60–80°. The extraction was continued with frequent stirring and intermittent heating to 60–80° for 4–5 days. The residue was separated by pouring through muslin and pressed, the turbid extract being filtered clear through paper. 90 cc. hydrochloric acid (1 pt. conc. HCl: 1 pt. water) were then added to the clear extract and the solution vigorously stirred. A characteristic precipitate of nucleic acid separated, quickly settling to a hard cake at the bottom of the vessel. After standing for two hours the liquid was syphoned off and the crude nucleic acid was washed with 50 % alcohol till free from chlorine, left standing over night in 95 % alcohol and finally washed with absolute alcohol and ether. The yield was 15 g. In other experiments the yields varied from 1·4–1·6 % of the dry alcohol-extracted yeast. The crude nucleic acid thus obtained

was a light brown powder and contained 7·8 % phosphorus. It gave no biuret reaction and had the characteristic properties of nucleic acid.

The crude substance was best purified by a method described by Osborne and Harris [1902] by solution in warm 10 % sodium acetate solution and precipitation with excess of hydrochloric acid. 20 g. of the crude acid were dissolved in 500 cc. 10 % sodium acetate by warming for a short time on a water-bath. A small amount of insoluble matter was separated by centrifuging and a clear solution obtained, to which 100 cc. alcohol and excess of hydrochloric acid were added. The nucleic acid separated out as a white mass which settled quickly. It was well washed with 50 % alcohol, 95 % alcohol and ether, and finally dried in a vacuum. It was a perfectly white powder. There is no loss in this method of purification which gives a much whiter product than purification from dilute potash.

The pure nucleic acid thus prepared gives no biuret reaction. Dried at 110° in air it gave the following results on analysis:

0·5626 g.; 0·189 g. $Mg_2P_2O_7$	P = 9·3 %
0·3905 g.; 45·9 cc. $N/10$ H_2SO_4	N = 16·4
Calculated for $C_{38}H_{50}O_{29}N_{15}P_4$	P = 9·5
	N = 16·1

5 g. of the above preparation were suspended in 200 cc. water and 100 cc. of a 0·1 % solution of caustic potash added. A clear solution was obtained from which the nucleic acid was precipitated by hydrochloric acid (20 cc. $N/2$ HCl). After the nucleic acid had separated, an equal volume of 95 % alcohol was added to the solution and the pure nucleic acid separated and washed with alcohol and ether in the usual way. Dried at 110° in air it gave on analysis the following results:

0·359 g.; 0·124 g. $Mg_2P_2O_7$	P = 9·6 %
0·2903 g.; 34·7 cc. $N/10$ H_2SO_4	N = 16·5

These results are in good agreement with the formula proposed by Levene [1909] for yeast-nucleic acid.

5 g. of pure nucleic acid were heated with 20 cc. of 10 % sulphuric acid solution on a boiling water-bath for two hours and strong ammonia in slight excess was added to the dark coloured solution. Guanine separated out on cooling as a dark coloured powder. It was purified by dissolving in dilute sulphuric acid, clearing with a little animal charcoal, and reprecipitating with strong ammonia. It gave a characteristic salt with hydrochloric acid and the usual reaction for guanine.

Preparation of nucleic acid from wheat embryos.

1000 g. of fresh wheat embryos (containing 0·78 % P) were boiled for two hours with 3 litres of 95 % alcohol, pressed free from the solvent and dried in air. The dry material was extracted with 10 litres of 10 % sodium chloride at 60–80° for five days[1]. The extract, which was somewhat viscid owing to the presence of gelatinised starch, was separated from the residue of wheat embryos by filtering through muslin and washing the latter with water, and was then centrifuged as clear as possible. 100 cc. hydrochloric acid (1 pt. conc. HCl : 1 pt. water) were added. Crude nucleic acid separated out and was washed with alcohol and ether in the usual way. Yield = 14 g. of a white powder containing a large amount of starchy impurity. It gave no biuret reaction and contained 4·6 % phosphorus which corresponded to approximately 50 % nucleic acid. In subsequent experiments yields varying from 1·1–1·4 % dry material were obtained.

50 g. of the crude nucleic acid (P = 4·6 %) was dissolved in 1000 cc. of a 10 % sodium acetate solution by gently warming on a water-bath. A large amount of starchy impurity remained undissolved and was easily separated by centrifuging. Nucleic acid was precipitated from the sodium acetate solution by the addition of excess of hydrochloric acid. The precipitated acid was washed with 50 % alcohol, 95 % alcohol and finally with ether. The yield of purified nucleic acid was 22·5 g.

5 g. of the nucleic acid thus purified was dissolved in 400 cc. 0·1 % caustic potash solution. A clear solution was obtained from which the nucleic acid was precipitated by adding 5 cc. of strong hydrochloric acid and an equal volume of 95 % alcohol. The precipitated acid was washed with alcohol and ether in the usual way and dried in a vacuum.

Dried at 110° it gave on analysis the following results:

0·3916 g.; 0·116 g. $Mg_2P_2O_7$ P = 8·3 %

0·329 g.; 45·2 cc. *N*/10 H_2SO_4 N = 16·1

It has been mentioned that loss of nucleic acid and considerable inconvenience was caused in this method of preparation by the presence of gelatinised starch in the extract. In subsequent preparations this difficulty was overcome by digesting the starch with taka-diastase before extracting the nucleic acid with sodium chloride solution.

1500 g. of wheat embryo (P = 1·13 %) were treated in the usual manner with boiling alcohol and dried in air. The air-dried material was then added

[1] The mixture was not kept constantly at 60–80°, but was heated up every day to this temperature and allowed to cool slowly.

in small portions at a time to 10 litres of boiling water and heating continued for two hours in a boiling water-bath. A thick paste was formed. This was transferred to two large flasks, cooled to 40° and a solution of taka-diastase containing 2·5 g. was added to each flask. The fermenting liquid was covered with toluene and the fermentation continued until no starch reaction with iodine was given. The liquid was then heated to 100° cooled to 60° and sufficient sodium chloride added to make a 10 % solution. The extraction with salt solution was continued for four days at 60–80° (intermittent heating). A perfectly clear extract was obtained by filtering through paper pulp and on pressing the residue practically the whole of the extract could be recovered. Nucleic acid separates from the clear extract in the usual characteristic way. Yield = 7 g.

The crude product was purified by dissolving in 400 cc. 0·1 % caustic potash solution, precipitating with hydrochloric acid, washing with alcohol and ether and drying in air. Dried at 110° it gave on analysis the following results:

0·3717 g.; 0·119 g. $Mg_2P_2O_7$ P = 8·9 %

0·3997 g.; 46·6 cc. $N/10$ H_2SO_4 N = 16·3

The authors desire to express their thanks to Mr A. E. Humpheries, Cox's Lock, Weybridge, and to Mr Julian Baker, F.I.C., Stag Brewery, Pimlico, for kindly supplying the raw material used in this investigation. They are also indebted to Messrs Parke, Davis and Co. for the supply of taka-diastase.

Summary.

General methods have been worked out for preparing protein-free nucleic acid from plant tissues without peptic digestion by first boiling the material used with alcohol and then extracting the alcohol-treated material with warm 10 % sodium chloride solution. Protein-free nucleic acid is precipitated from the sodium chloride extract on the addition of hydrochloric acid.

In the case of material containing starch, such as wheat embryos obtained in the modern processes of milling, the starch was removed by fermentation with diastase before extracting with sodium chloride solution. In this way extracts were obtained which could easily be filtered.

The analytical results obtained were in good agreement with the formula $C_{38}H_{50}O_{29}N_{15}P_4$ suggested by Levene for plant nucleic acid.

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XXX. A COMPARATIVE STUDY OF TUMOUR AND NORMAL TISSUE GROWTH.

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Introduction.

From early times the profound influence exerted upon the nutrition of an animal by the composition of its diet has been recognised, so that it is not surprising that a vast amount of research has been carried out upon the subject.

Whilst much of this research has furnished results of great value, it is as a result of the investigations of the last decade in particular that the theoretical basis of the science of nutrition has become so firmly established.

These results have permitted a far clearer definition of the many factors which control the nutritive value of a foodstuff than it was ever possible to deduce from calculations respecting the calorific value and nitrogen content.

Particular interest lies in the determination of the dietary factors which influence the growth and development of the young organism. Many of these factors are now recognised to such an extent that by an experimental application of them the growth of young animals may be controlled. This control may be so complete as to permit the total inhibition of the growth processes for a considerable period of time, without the subject suffering any apparent ill-health.

It is here that such results may be viewed in the light of cancer therapy, since the question arises as to whether dietary measures which inhibit the growth of the young cells of the animal organism may not be with justification applied as a means of arresting growth in tumour cells.

Tumours are frequently described as autonomous, in that their growth is self-regulated without regard for the laws governing the nutritive condition of the host. On the other hand there are facts, such as the relatively slow

growth of tumours in old or emaciated hosts, which lend support to the opposite view.

It is not easy to trace opinions, based upon clinical observations, which concern this subject, since they are scattered widely apart throughout the literature upon cancer.

Before proceeding to discuss the laws of the growth of tumours, it is necessary that the known facts concerning their metabolism be considered. Unfortunately, comparatively little is known with regard to the metabolic processes of the cancer cell. From what is known, it appears highly probable that there is a close similarity between the chemical processes occurring in the tumour cell and those which occur in the normal cell of similar type. It is unnecessary to give in detail an account of the experimental results which support this view, but Wells [1914] who has reviewed the whole subject very completely, expresses the opinion that little has been detected which indicates any important deviation of the chemical processes of tumours from those of normal cells of similar origin.

As this opinion has received the support of the majority of the experimental investigations since carried out, it will be seen that it is justifiable to apply factors which are known to influence the growth of normal cells in an attempt to influence in a similar manner the growth of tumour cells.

It is now necessary to review briefly what is known of these factors which may so profoundly influence the nutritional condition of the animal organism.

It is recognised as necessary that the diet of an animal shall satisfy certain requirements before it can be regarded as adequate to supply the demands of the organism throughout its life cycle.

As far as these requirements have been determined they are as follows:

1. The calorific value of the diet must be sufficient to supply the necessary potential energy.

2. Sufficient nitrogen must be supplied in a form suitable for the building up or repair of tissue.

3. This nitrogen must be supplied in a form which will ensure an adequate supply of certain amino-acids which the animal organism is unable to synthesise for its own use.

4. The diet must contain an adequate supply of inorganic salts capable of satisfying the mineral requirements of the animal.

5. Certain substances, probably two in number, the nature of which is at present unknown, but which have been provisionally termed "accessory growth promoting substances," must be present in a sufficient amount.

With regard to the first two of these requirements little need be said, for until recently they, together with the fourth stipulation, constituted the basis for the standardisation of the nutritive value of foodstuffs. The value and importance of the other two factors has, however, but recently been appreciated. Prior to 1911, repeated attempts had been made to rear and maintain animals upon dietaries composed entirely of chemically pure food units, but although every care was taken to ensure that the diets used contained satisfactory proportions of protein, fat, carbohydrates and mineral salts, yet such experiments met with repeated failure. It was Osborne and Mendel [1911] who first described an extensive research conducted along these lines which was attended with considerable success.

The results obtained by these two authors were of far-reaching importance and opened up a new field of research on protein metabolism.

Earlier studies than these had, however, determined the importance of the quality of the protein fraction of a diet, for Willcocks and Hopkins [1906] demonstrated the inferior nutritive value of the protein zein, which is totally deficient in the amino-acid tryptophan. Osborne and Mendel followed up their investigations along the lines they had previously adopted, and not only confirmed the indispensability of tryptophan [1912, 1] but also pointed out how the amino-acid deficiencies of such a protein as gliadin may influence the nutrition of an animal [1912, 2, 3; 1913]. Much of this earlier work has now received ample confirmation which, together with the results obtained by an extension of the study, have been the cause of very material alterations in the theory of protein metabolism.

That the nutritive value of a protein may be largely determined by its amino-acid content is now so fully appreciated as to have passed the stage when it was regarded as of academical interest alone, and to have become of great value when applied to certain problems of animal husbandry.

Not only may the nutritive value of protein be greatly influenced by the absence of certain amino-acids, but it may be limited by a low content of one such indispensable unit. The case of gliadin, which possesses a low nutritive efficiency as a result of a low lysine content has been referred to above, whilst a very striking example has been given more recently by Osborne and Mendel [1915, 1].

When the diet of a growing rat contains 18 % of protein in the form of caseinogen or lactalbumin, and is at the same time satisfactory with regard to the other requirements, a normal rate of growth is accomplished. Upon reduction of the plane of protein intake, it is found that a diet containing

9 % of caseinogen is of a lower food value to the growing rat than one containing the same percentage of lactalbumin. The explanation of this was found to lie in the fact that caseinogen has a low content of cystine, an amino-acid which the organism of the rat is unable to synthesise.

At the lower plane of intake, the caseinogen diet contained insufficient protein to supply the cystine requirements of the young rat, whereas no such limiting factor was in operation in the case of the lactalbumin diet. That this explanation was the correct one was proved by the addition of the pure amino-acid cystine to the deficient caseinogen diet, whereupon its nutritive value was at once raised to that possessed by the corresponding lactalbumin ration.

Such experiments as these have led to the establishment of an entirely new standard by which the food value of the proteins is judged [Osborne and Mendel, 1916, 1].

The necessity of ensuring an adequate supply of what are termed the "accessory growth promoting substances" in the diet, particularly that of a growing animal, is but now being fully recognised. That such substances existed and played important rôles in animal nutrition was indicated by the researches of Stepp [1909, 1912, 1913], Hopkins [1912] and Funk [1913, 1]. Their existence has been doubted by other investigators, amongst whom may be mentioned Abderhalden [1913, 1] and Röhmann [1916], but it is definitely established that in these cases the authors were not utilising food mixtures of sufficient purity. These substances have received many appellations and are frequently spoken of as "vitamines."

At first one of these substances was found in association with certain naturally occurring fats, such as butter fat and cod liver oil, by Osborne and Mendel [1912, 1] and almost simultaneously by McCollum and Davis [1913].

Later it was demonstrated by McCollum and Davis [1915, 1, 2, 3] that a second accessory exists in the aqueous extracts of certain foodstuffs, particularly seeds.

These authors have shown clearly the importance of these two unidentified substances which they have provisionally designated *the fat-soluble A* and *the water-soluble B*. Both substances are at present unidentified chemically, and indeed very little definite knowledge of their properties is possessed. It appears highly probable that the second substance is identical with the so-called "anti-beriberi vitamine," studied extensively by Funk, since they possess closely similar properties, Drummond [1917]. The need of the animal organism for an adequate supply of both these factors is now admitted by the majority of investigators in this field of research.

Having now very briefly considered the nature of the requirements which a dietary must satisfy before it can be considered adequate to supply the nutritive demands of the animal organism, the recorded experiments upon the influence of diet upon tumour growth will be considered in the light of this knowledge.

Haaland [1907] recorded that mice fed upon a diet of bread, oats, hempseed and milk were less resistant to inoculation with Ehrlich's mouse sarcoma than when fed upon bread and oats alone. This observation received support from Stahr [1908], who attributed a different degree of susceptibility to tumour inoculation exhibited by mice from Berlin and those from Düsseldorf to an influence exerted by differences in their diets. A somewhat similar conclusion was reached by Jensen [1909]. In all these cases, however, the explanation given by the authors was that which seemed best to account for the results, but no experimental proof was given which excluded the possible disturbing influence of other factors.

It was Moreschi [1909] who first attempted a study of the influence of diet upon tumour growth. He found that by underfeeding mice the growth of inoculated grafts of tumour tissue, as yet unvascularised, was retarded. Rous [1911, 1] made a similar study to this, but also examined the influence of such dietary measures applied after the tumour grafts had become established in the host. He obtained the interesting result that whereas a tumour may not take well when implanted into an underfed animal, yet the same tumour may show no retardation in growth as a result of dietary restriction applied after vascularisation of the graft.

Sweet, Corson-White and Saxon [1913] investigated the influence upon tumour growth exerted by a diet in which the protein was supplied in form of gluten. Such a diet had been found by Osborne and Mendel [1912, 1] to permit maintenance, but not growth, in rats owing to its low content of lysine.

Sweet, Corson-White and Saxon aimed at determining whether a cancer could grow in a body rendered incapable of normal cell-growth. They found that the number of successful inoculations of grafts of the Flexner-Jobling rat carcinoma was smaller in the animals which had been fed upon the gluten diet than in the normally fed controls. At the same time the grafts which succeeded in becoming established in the specially fed animals exhibited a slower rate of development and more frequently suffered retrogressive change than in the control series.

They were therefore led to suggest that tumour cells and somatic cells agree with respect to their laws of growth.

Van Alstyne and Beebe [1913] did not agree with the conclusion reached by Sweet, Corson-White and Saxon. They themselves examined the influence of carbohydrates upon tumour growth in rats, and found that a greater degree of success in tumour implantation was obtained in the animals which received a diet containing carbohydrate, as compared with those which received a carbohydrate-free diet.

It is unfortunate that these authors should have chosen lactose as the carbohydrate with which to work, for it has been pointed out that lactose is frequently contaminated with traces of nitrogenous impurities. The possible influence of these impurities upon the course of such experiments as were carried out by van Alstyne and Beebe has been pointed out by Funk [1914, 1] and by Sweet, Corson-White and Saxon [1915].

In spite of this, Woglom [1915] challenged the conclusion of van Alstyne and Beebe, since he found no effect upon tumour growth following the administration of lactose to the hosts. What was probably the correct explanation of these two diverse results was given by Drummond [1916, 1] who pointed out that lactose, unless carefully purified, usually carries traces of the water-soluble accessory factor, *B*. It was probably this factor which influenced tumour growth in the experiments of van Alstyne and Beebe, whereas no such influence would be exerted in Woglom's experiments since he added the lactose to a basal diet already rich in accessory substances.

Rous [1914] followed up his earlier studies and extended his investigations to the more practical determination of the influence of diet upon established tumours in mice. Rous appreciated the doubtful practical value of results such as those obtained by Sweet and his co-workers, and by van Alstyne and Beebe. Commenting upon the lower rate of tumour development obtained in the hosts which had been subjected to dietary measures prior to inoculation, he says, "Unfortunately it is not certain whether the results of these investigations are to be attributed to a specific lack in the foods employed or to the circumstance that the diet of the specially fed hosts differed from that of the animal furnishing the tumour transplanted to them." He therefore conducted a most important investigation in which he studied the effects of dietary restrictions upon spontaneous tumours in mice.

By underfeeding mice upon a gluten ration, similar in composition to that employed by Sweet, for several days prior to operation, the development of recurrences, following incomplete removal of the primary tumour, was in many cases considerably delayed. If, however, the special diet was employed after operation this delay was not brought about. Unoperated spontaneous

tumours seemed unaffected by rigorous dietary restriction and no cures were obtained in any dieted animal.

In the same paper he gives evidence which shows that the reactionary processes of the organism are considerably weakened by the malnutrition which results from the dieting. It therefore appears probable that this fact may furnish the explanation of the lower percentage of successful inoculations in poorly nourished hosts reported by Sweet, and by van Alstyne and Beebe.

Reviewing his work Rous [1915] has remarked, "Special experiments have shown that our results are to be attributed solely to the underfeeding and resultant loss of body weight, and not to the character of the food. The treatment was drastic. The best results were obtained with animals losing weight rapidly at the time of operation." In view of these results he does not feel justified in advocating the employment of underfeeding as a palliative treatment of cancer.

Following a somewhat different line of investigation Funk [1914, 1, 2] has made a study of the influence of what he terms "vitamines" upon the growth of a transplantable sarcoma of the fowl. Unfortunately his results must be regarded as inconclusive, chiefly as there is considerable doubt as to whether, under the conditions employed for the experiments, the chicken is a satisfactory subject for such studies [Drummond, 1916, 2].

Benedict and Rahe [1917] have recently investigated the influence of the "vitamines," or accessory growth factors, upon tumour growth. They employed dietaries similar in composition to those which have been used by Funk and Macallum [1915, 1916, 1, 2], and determined that tumour cells had no power to synthesise the accessory substances when these were absent from the diet of the host, but that at the same time a certain amount of tumour growth occurred under these circumstances, at the expense of the tissues of the animal.

From this brief summary of the more important work which has been carried out upon this subject, it will be realised how little is known regarding the influence of diet upon tumour growth. This is largely due to the disconnected character of the researches, and to the difficulty of co-ordinating the results they have yielded.

Few of these investigations can be said to have a close bearing upon the cancer problem, for the determination of the fact that underfeeding an animal will render it less susceptible to tumour inoculation scarcely applies to any phase of the disease in man. A low state of nutrition, whether induced by

poverty in the diet or by an intercurrent disease, is known to have an unfavourable influence upon tumour implantation.

Theoretically, all such studies as these should be carried out upon animals bearing spontaneous tumours, for they alone are to be regarded as the equivalent of the cancer patient. Rous has carried out a study upon such animals, which has already been referred to, but it is seldom that the opportunity of working with large numbers of these subjects presents itself.

The nearest approach to this ideal condition of cancer research is to study the implanted tumour after it has become established in the body of the host. It must be admitted that even this substitute for the cancerous animal falls regrettably short of what is desired, but it is at present the best available.

The present investigation was designed to study the influences exerted upon tumour growth by the following dietary factors.

(*a*) The plane of protein intake of the host.

(*b*) The character of the protein constituents of the diet of the host.

(*c*) The amino-acid content of the diet.

(*d*) The so-called accessory factors, the *fat-soluble A* and the *water-soluble B*.

Experimental.

Methods.

Rats were chosen as the subjects for this experimental research on account of their great suitability for such work. The great majority of the animals employed were of the albino variety of *Mus norvegicus*, but a few of the black hooded variety of the same animal were also used.

A large number of the experimental animals were those bred from the healthy laboratory stock, whilst others were purchased from reliable breeders.

All stock rats were kept under the closest observation during the period before they were used for experimental purposes. This provided the opportunity of weeding out unsuitable specimens. The general health of the stock was, however, so excellent that the number of rejected animals was very low indeed. As soon as batches of the selected animals had reached a suitable size and weight they were inoculated with the tumour under investigation and returned to the pens.

They were again closely observed during the initial stages of the development of the inoculated graft, until a number of them showed well established and actively growing tumours. These animals were then placed in special boxes and used for dietary experiments. The boxes used for these

experiments were shallow wooden ones with wire-covered tops and sawdust-sprinkled floors. At one end was placed an enclosed sleeping-box of a size proportionate to the number of occupants. All these boxes were cleaned out at least every three days, whilst occasionally the inmates were transferred to new and thoroughly cleansed quarters.

The experimental rats were fed every morning and evening with fresh supplies of the various food preparations. These latter were prepared by thoroughly incorporating the ingredients in a mechanical mixer and adding sufficient water to form the whole into a very stiff paste. This was then made up into small hard balls weighing approximately 5 grams apiece, in which form it was supplied to the animals. By this means it was possible to make a rough estimate of the twenty-four hours' food consumption. Small quantities of the diets were prepared at one time and stored at the temperature of the refrigerator room (1·1°), as the superior value of freshly prepared food mixtures was found to repay the trouble which the more frequent preparations entailed.

All experimental animals were subjected to the closest observation. Body weights were recorded every four days and tumours were charted once a week, whilst at the same time occasional observations on the body temperature were registered.

The tumours utilised for this work were two rat sarcomas which are designated for laboratory purposes, *A* and *S*.

The former was used in some of the earlier studies, but its employment was discontinued owing to certain characteristics it possessed which rendered it less suitable for the purpose than the *S* tumour.

The *A* tumour is a round celled sarcoma. Grafts inoculated into suitable rats usually develop satisfactorily in from 60—80 % of the cases. The tumour grafts generally show a rapid rate of growth, but the resulting tumours are particularly prone to early and extensive necrotic changes. It is frequently found that after a week or two's growth the whole central portion of the tumour is so extensively degenerated that there remains but a thin shell of growing tissue surrounding a mass of necrotic cell debris.

It was owing to this characteristic that the use of this particular tumour was discontinued and the *S* tumour adopted. The latter tumour is a spindle-celled sarcoma showing a high degree of virulence. Inoculated grafts in suitable hosts usually develop satisfactorily in 85—100 % of the cases. It exhibits a slightly slower rate of growth on the average than does the *A* tumour, but it is not nearly so prone to degenerative changes. The *S* tumour

remains firm until a relatively late stage in its development, and when necrotic changes in the centre of the growth do occur they do not extend rapidly.

Neither tumour is accompanied during its growth by microscopic evidence of the formation of metastases when the grafts are subcutaneous.

The inoculation of the rats was carried out by implanting small pieces of tumour tissue, of approximately equal size, subcutaneously in the neighbourhood of the right axilla by the trochar method. The question of the importance of what is termed the effective initial dose [Bashford, Murray, Haaland and Bowen, 1908] was considered before this method of inoculation was decided upon.

Several methods have been elaborated which aim at ensuring a closer approximation to uniform dosage at inoculation, but it is uncertain, having due regard to the more or less uncontrollable influences exerted by those factors which determine the susceptibility of the host, whether these methods are successful in attaining their object. In view of this uncertainty, the method of inoculating small pieces of tissue of an approximately equal size was considered sufficiently accurate for the purposes of this research. No animal which failed to show a satisfactory rate of tumour development during the period of observation following the inoculation was used for feeding experiments. All animals at the conclusion of the experiments were subjected to a post-mortem examination, at which pieces of the tumours and certain organs were removed and fixed in Zenker's fluid, for subsequent microscopical examination.

The mortality amongst the experimental animals from intercurrent disease was exceedingly low, only one or two cases occurring throughout the investigation.

The preparation of the individual components of the various dietaries was carried out by the processes described below.

Caseinogen was prepared from diluted skimmed milk by acidification as described by Osborne and Mendel [1911]. The crude protein was twice reprecipitated from solution in dilute alkali by the same method, and was then extracted by being shaken vigorously in repeated changes of distilled water, the first two changes containing a trace of acetic acid. The resulting product was dehydrated and repeatedly extracted with alcohol and ether at room temperature.

Lactalbumin was prepared by heat-coagulation of the proteins present in the faintly acidified filtrate from the precipitated caseinogen obtained in the last preparation. It was purified by several extractions with boiling

distilled water and subsequent treatment with changes of boiling alcohol and ether.

Edestin, zein and gliadin were prepared from hempseed, crushed maize, and wheat respectively by the processes described by Osborne [1910]. They were in each case subjected to careful purification, as directed by this author, to ensure preparations of a high degree of purity.

Ovalbumin was prepared from egg-white according to the process of Hopkins and Pinkus [1900]. It was a crystalline product of considerable purity.

Gelatin was that supplied by Coignet Père et Fils et Cie and bore the mark "Gold label, extra." It gave at the most only a faint coloration with Millon's reagent.

Starch was purchased as pure wheaten starch. It was submitted to two extractions with boiling alcohol before being included in the dietaries.

It is not out of place to discuss at this juncture the process of alcohol extraction which is somewhat commonly used to render foodstuffs free from traces of the accessory factors. Stepp [1909, 1912] found that alcohol extraction removed indispensable substances from natural foodstuffs, and it has been used extensively for this purpose by Funk [1914, 1] and others. From recent work, however, it appears that in the absence of water absolute alcohol will not dissolve the water-soluble accessory substance [Osborne and Mendel, 1917; Drummond, 1917]. It is therefore possible that alcohol extraction does not remove traces of this substance when the foodstuff is extracted in the dry state.

This point was, however, only determined after the present investigation was completed.

Agar was included in the majority of rations to provide an indigestible intestinal ballast or "roughage." It was purchased in the powder form and was subjected to the somewhat empirical "purification" by hot alcohol extraction.

Sucrose was not subjected to any further purification.

Lactose was added to some dietaries, not only to serve as a source of carbohydrate, but also as a means of adding a certain amount of the water-soluble growth accessory factor, which it usually contains [McCollum and Davis, 1915, 1; Drummond, 1916, 1]. In some instances the lactose used was a crude preparation, very rich in this unidentified dietary factor, which was very kindly supplied by the courtesy of "Casein, Ltd." It was a light sandy coloured crystalline powder representing the first crystalline fractions obtained in the commercial preparation of lactose.

The nitrogen content of specimens of lactose is to a large extent a measure of their impurity. A large number of samples of this sugar were analysed for nitrogen. Preparations sold as pure lactose were found to contain from 0 to 0·02 %. The nitrogen-free specimens were usually preparations consisting of large crystals, and were found to be uncontaminated by the presence of the water-soluble growth factor.

The crude lactose referred to above was found to contain 0·19 % nitrogen.

"*Protein-free milk*" was used as a source of lactose, inorganic salts and the water-soluble growth promoting factor in some rations. It was prepared as described by Osborne and Mendel [1911], and was a sandy coloured crystalline powder having a nitrogen content varying between 0·57—0·67 %.

The use of "protein-free milk" in artificial rations has not escaped criticism upon the grounds of its indefinite composition [Funk, 1914, 1; Funk and Macallum, 1914; McCollum and Davis, 1915, 4]. Whilst there is undoubtedly some justification for these criticisms, it must be recognised that the use of this product has greatly assisted the elucidation of many important points concerning the nutritive value of the proteins [Osborne and Mendel, 1916, 1, 2].

Lard was used as a source of fat when it was desired that the diet should be free from the fat-soluble *A* accessory substance, since it has been repeatedly shown that lard does not contain this substance.

Butter fat was used as a source both of fat and of the fat-soluble accessory factor. It was prepared by centrifugalisation of butter at a temperature just above its melting point, and separation of the clear fatty layer. Determinations of nitrogen by the Kjeldahl process indicated that the butter fat was nitrogen-free. There is, however, some uncertainty whether this fat does not usually carry traces of nitrogen and phosphorus [Funk and Macallum, 1914; McCollum and Davis, 1914; Osborne and Wakeman, 1915].

"*Yeast Preparation*" was used in a number of dietaries as a means of ensuring an adequate supply of the water-soluble growth accessory. The preparation was made by evaporating down to a thick syrup the aqueous dialysate of a commercial yeast extract (marmite), at low temperature and reduced pressure.

The resulting pale brown syrup is very rich in the water-soluble accessory substance. It also contains relatively large amounts of adenine, leucine, sodium chloride and potassium phosphate [Drummond, 1917].

Amino-acids were prepared by the usual methods from various protein hydrolysates. Tryptophan was obtained from the tryptic digest of caseinogen.

Histidine from hydrolysed ox-blood, lysine and arginine from hydrolysed gelatin, tyrosine from silk waste, and cystine from hair. In the case of the last two preparations the amino-acids tyrosine and cystine were freed from traces of each other by the phosphotungstic acid process described by Plimmer [1913].

Hydrolysed Meat-Protein. Fresh lean beef was minced and extracted three times with boiling water, which was slightly acidified with acetic acid at boiling point to complete the coagulation of proteins. The residue of tissue protein was well pressed out in a meat press and both dehydrated and extracted by treatment in a continuous extraction apparatus firstly with alcohol and subsequently with ether.

The resulting product, after being ground and passed through a fine mesh sieve, was submitted to hydrolysis with boiling 15 % sulphuric acid for 48 hours. From the resulting deep brown coloured fluid sulphuric acid was removed quantitatively by the careful addition of boiling saturated barium hydroxide solution. The bulky precipitate of barium sulphate, contaminated with much pigmented matter, was extracted by boiling with several changes of distilled water. The combined washings were united with the main filtrate and the whole was evaporated down until a thick syrup was formed. Extracted wheaten starch was then added until a stiff paste was obtained, which was spread out in a thin layer over heated glass plates and dried until it could be powdered and passed through a fine meshed sieve.

This powder possessed a pale brown colour and contained approximately 7 % of nitrogen. To render this product nutritively the equivalent of the original protein it was necessary to replace the destroyed tryptophan. This was done by adding 0·7 % of the pure amino-acid prior to the incorporation with starch.

Hydrolysed Meat-Protein, Monamino-acid fraction. Beef protein was submitted to acid hydrolysis as described in the preceding preparation. To the neutral filtrate after removal of the sulphuric acid was added sufficient of a 20 % solution of sulphuric acid to render the whole about 5 % in strength. A 30 % solution of phosphotungstic acid was then added until no further precipitation occurred. The heavy precipitate was allowed to stand for 24 hours at 2°, being then filtered off and well washed with an ice-cold 5 % solution of sulphuric acid. The filtrate and washings were quantitatively freed from the excess of sulphuric acid and the precipitant by the careful addition of baryta, evaporated down and incorporated with starch as described in the previous preparation.

The final product was a pale sandy coloured powder containing approximately 5 % of nitrogen.

"*Erepton.*" I was fortunate in being able to secure several hundred grams of a commercial preparation of hydrolysed meat-protein, placed on the market by Meister, Lucius and Brüning.

The product was labelled "Erepton. Vollständig abgebautes Fleischeiweiss, nach Prof. Dr Abderhalden." It consisted of a brown, granular, slightly hygroscopic mass and contained from 12·5—12·8 % of nitrogen. As far as could be ascertained the degradation of the protein had been completed. I have to thank Mr E. Scholl, of John George Haller and Co., for his great kindness in placing this preparation at my disposal.

Salt mixture. Inorganic salts were supplied in some of the rations in the form of a salt mixture possessing a composition identical with one of those used by McCollum in his extensive researches. It was made up as follows:

NaCl	1·73 g.	$CaH_4(PO_4)_2$, $2H_2O$	5·40 g.
$MgSO_4$ (anhydrous)	2·66 g.	Calcium lactate	13·00 g.
NaH_2PO_4, H_2O	3·47 g.	Ferric lactate	1·18 g.
K_2HPO_4	9·54 g.		

Traces of iodine were supplied in the drinking water once a week, as recommended by McCollum.

Alcoholic Extract of Dried Milk. This preparation has been extensively used by Hopkins and his co-workers [Ackroyd and Hopkins, 1916] as a means of adding to the dietary a sufficient amount of accessory substances.

The use of this preparation during this work has not been found as satisfactory for this purpose as the employment of the combination of butter-fat and yeast preparation. This is probably explainable by the fact that the water-soluble substance is insoluble in absolute alcohol, and unless the milk powder contains sufficient moisture, the extraction of this substance is liable to be incomplete. The results of the series of experiments carried out during this investigation will now be considered in detail.

The influence of the level of protein intake of the host upon normal and tumour growth.

Much important work has been carried out recently upon the influence which the protein content of the diet may exert upon the nutrition of the animal. The papers by Osborne and Mendel [1912, 2; 1915, 1, 2; 1916, 1], and Janney [1915] contain results of importance dealing with this subject.

From these results it is now known that a reduction in the level at which protein is furnished in the diet of a young animal will sooner or later induce a more or less complete inhibition of the growth processes of that animal. This will occur either when the nitrogen intake falls below the level required

	Days 14	21	28	35	42	Remarks	Weight of tumour g.	Weight of host - tumour g.	Change in weight of host g.	Change in weight of host per 1 g. tumour
♂						Tumour solid and rapidly growing	25	126	+30	
♂						Slight central necrosis of tumour	27	140	+37	
♂						—	15	132	+39	
♂						Tumour had ulcerated and undergone central necrosis	19	120	+24	
♀						—	22	130	+36	
♀						Tumour retrogressed	–	152	+44	
						Average	21·6	–	+35	+1·62

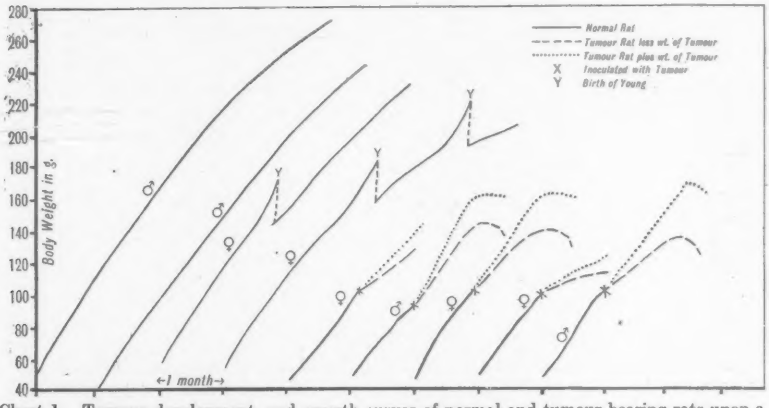
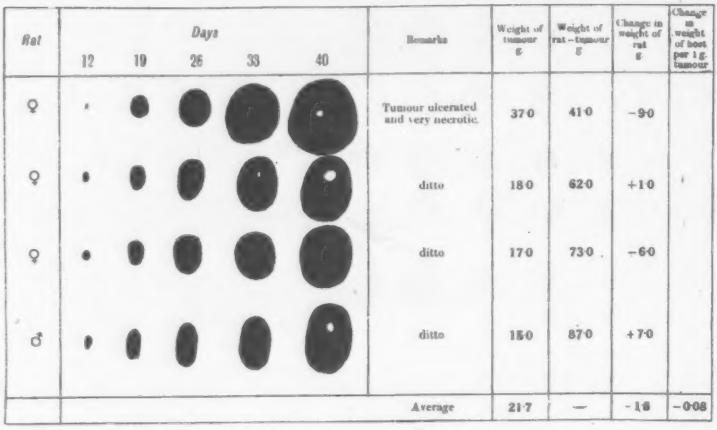


Chart 1. Tumour development, and growth curves of normal and tumour-bearing rats upon a normal diet of bread, oats, corn, greenstuffs and meat. Tumour *S*/113*a*.

to supply the demands of the organism, or when the lowered protein intake brings about a deficiency in the supply of one or more amino-acids which are indispensable to the animal. Examples of how this may occur have been given in the introduction to this paper. A large number of proteins, par-

ticularly those of vegetable origin, possess amino-acid contents which limit their nutritive value to no inconsiderable extent [Mendel, 1915].

Chart 1 serves as a control to a number of the experimental results of this work, for it illustrates the growth of young rats, the growth of tumour-bearing rats and the development of the tumours (*S*) they carry, upon a normal mixed diet of bread, seeds and greenstuffs, with occasional additions of meat or milk. The normal growth of the *A* sarcoma is given in Chart 2.



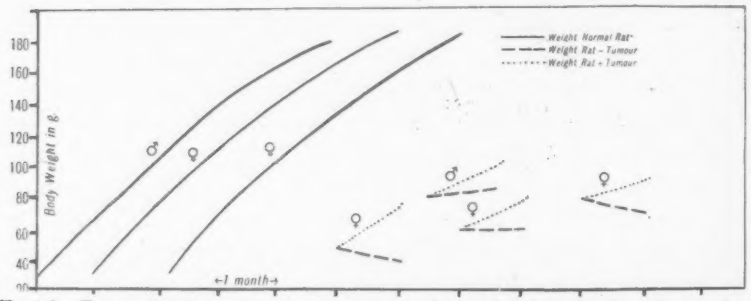


Chart 2. Tumour development, and growth curves of normal and tumour bearing rats upon complete artificial diet. Tumour *A*/30*d*. Diet: Caseinogen, 18 %, butter-fat, 20 %, "Protein-free milk," 30 %, Agar, 5 %, Starch, 27 %.

Chart 3 indicates the results which were obtained by feeding a "complete" artificial dietary composed as given below:

Caseinogen	18 %	Salt mixture	5 %
Starch	48 %	"Yeast preparation"	6 %
Agar	3 %	Butter-fat	20 %

It will be seen from Chart 3 that this diet is adequate to supply the nutritive requirements of the rat throughout its life cycle.

Tumour development proceeds at an equally normal rate in hosts fed upon this dietary, and is not accompanied by a loss in weight of the animal itself, until secondary disturbances, such as an ulceration of the tumour with resulting sepsis, set in.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour firm and soled	10·7	11·3	+17·3	
♂						Slight central necrosis	25·5	140·5	+40·5	
♀						Animal died 17.4.17 Tumour firm	6·7·	141·3	+55·3	
♀						Central necrosis	24·0	146·0	+55·0	
♀						—	16·0	127·0	+42·0	
♂						Tumour retrogressed	—	—	—	
						Average	16·6	—	+42·0	+2·5

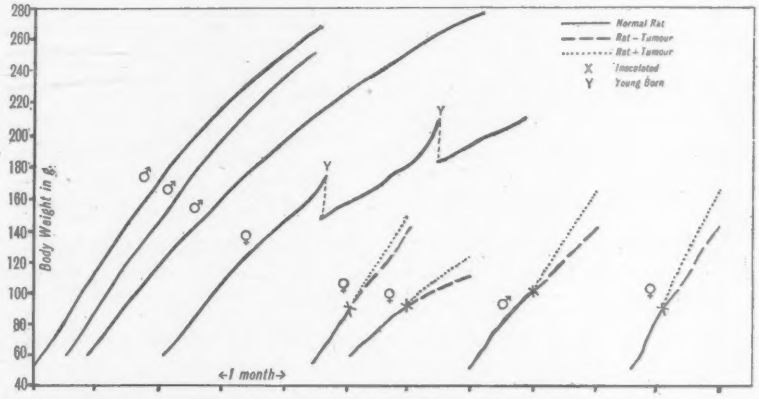


Chart 3. Tumour development, and growth curves of normal and tumour-bearing rats upon a complete artificial dietary. Tumour *S*/120*d*. Diet: Caseinogen, 18 %, Starch, 40 %. Agar, 3%, Salt Mixture, 5 %, "Yeast preparation" 6 %, butter-fat, 20 %.

As has already been stated, Osborne and Mendel [1915, 1] found that when the caseinogen content of such a diet as the above was lowered, a stage

was eventually reached where the low cystine content of the caseinogen became a factor limiting the growth processes of the animal. Accordingly the effect of a similar reduction in the protein content upon the growth of tumours was investigated. Two diets were compounded containing respectively 10 and 6 % of caseinogen, but in other respects possessing a composition similar to the diet described above. The results obtained in feeding these rations to the normal young rats support the work of Osborne and Mendel, although growth inhibition appears to have become marked at an earlier stage in the reduction of the protein level (Charts 4 and 5).

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♂						Much central necrosis of tumour	27·0	58·0	−23·0	
♀					–	Animal died 7. 1. 17. Tumour very necrotic and ulcerated.	14·0	63·0	−15·0	
♀						Firm tumour	8·5	60·5	−11·5	
♂						ditto	5·5	112·5	−17·5	
♀					–	Died 29. 12. 16. Tumour firm.	8·8	65·0	−15·0	
♂					–	Tumour retrogressed	–	–	–	
						Average	12·2	–	−16·4	−1·35

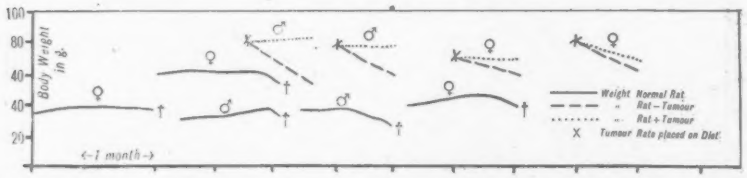


Chart 4. Tumour development, and growth curves of normal and tumour-bearing rats upon low protein diet. Tumour *S*/117*c*. Diet: Caseinogen, 6 %, Starch, 48 %, Agar, 3 %, Salt mixture, 5 %, "Yeast preparation" 6 %, Butter-fat, 20 %, Sucrose, 12 %.

The results yielded by the tumour-bearing animals may be conveniently summarised in tabular form.

TABLE 1.

% of caseinogen in diet	Average weight of tumours, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. tumour
18	16·6	+42·0	+2·50
10	16·0	−19·4	−1·21
6	12·2	−16·4	−1·35

No evidence of tumour retardation is apparent in the case of the animals fed upon the 10 % caseinogen diet, but tumour growth is in these cases accompanied by a somewhat serious drop in the body-weight of the host. Where the diet contained 6 % of the protein there is a small but appreciable decrease in the size of tumour growth, also accompanied by a drop in the body-weight of the host. It will be noticed that the curves representing the weight of the

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Slight central necrosis	29·0	46·0	−26·0	
♀						Tumour firm	25·0	53·0	−17·0	
♀						ditto	14·0	38·0	−11·0	
♀					–	Animal died 29. 12. 16. Tumour firm	8·5	51·5	−24·5	
♀							3·5	96·5	−18·5	
♂						Tumour retrogressed	–	–	–	
						Average	16·0	–	−19·4	−1·21

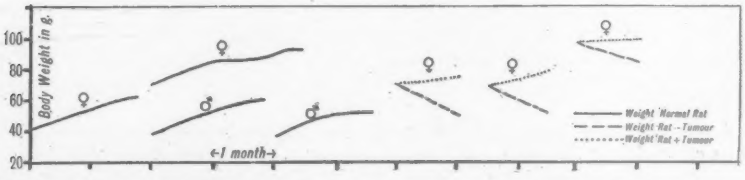


Chart 5. Tumour development, and growth curves of normal and tumour-bearing rats upon low protein diet. Tumour *S*/117*c*. Diet as in Chart 4, but containing 10 % of Caseinogen.

host plus tumour run roughly parallel to those which indicate the growth of the normal animal upon the same diet, whilst the weight of the host alone shows a gradual fall. This makes it probable not only that the food requirements of the tumour are satisfied before those of the host, but also, that if necessary the tissues of the host are drawn upon to supply deficiencies in the former.

Chart 6 illustrates the results of feeding a diet containing a low level of

protein to normal and tumour-bearing animals. The diet was composed as given below:

Dried whole-milk powder	15 %	Salt mixture	3 %
Starch	49 %	Butter-fat	10 %
Agar	3 %	Sucrose	20 %

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat—tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♂						Tumour ulcerated and very necrotic.	25·0	55·0	−24·0	
♂						Tumour very necrotic	20·0	82·0	−29·0	
♀						Ulcerated and necrotic	15·0	35·0	−29·0	
♂						ditto	16·0	74·0	−41·0	
♂						Tumour much degenerated	11·0	69·0	−22·0	
♀							3·0	59·0	−29·0	
						Average	15·0	–	−29·0	−1·94

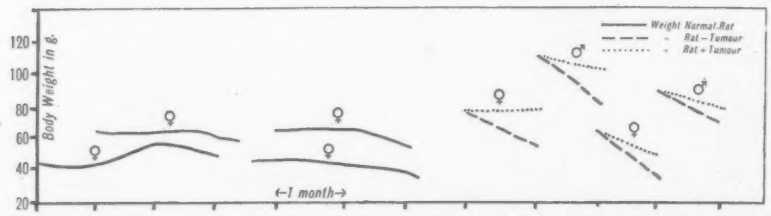


Chart 6. Tumour development, and growth curves of normal and tumour-bearing rats upon low protein level diet. Tumour *A*/24*a*. Diet: Dried milk, 15 %, Starch, 49 %, Agar, 3 %, Salt mixture, 4 %, Butter-fat, 10 %, Sucrose, 20 %.

Calculating from the known composition of the dried milk powder, this diet contained approximately 5 % of milk protein. Normal young rats fed upon this diet remain in good health, but show no change in body-weight for several months. The nitrogen balance and body temperature remain normal during this period of growth inhibition. Tumour growth in rats fed upon this same diet is however a little retarded, as is shown by the average weight of the tumours (15 g.) compared with the average weight (21 g.) grown on the normal dietary. A severe loss of body-weight upon the part of the host on the low protein diet may be observed in these cases also.

The influence of the character of the protein present in the diet upon normal and tumour growth.

Any consideration of the results of the preceding section of this paper cannot be dissociated from a study of the results given in this one, since they are so closely interrelated.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour very necrotic and ulcerated.	31·0	59·0	−1·0	
♀						Tumour necrotic	12·0	68·0	+14·0	
♀						ditto	9·0	83·0	+13·0	
♀						ditto	10·0	90·0	−9·0	
						Average	15·5	—	+4·2	+0·27

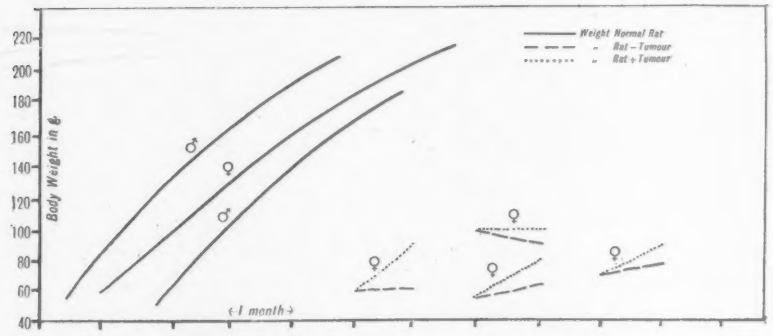


Chart 7. Tumour development, and growth curves of normal and tumour-bearing rats upon Edestin diet. Tumour *A*/30*d*. Diet as in Chart 2, but Edestin replacing Caseinogen.

The relative nutritive values of the proteins have already received attention in the introductory section, so that the results which are illustrated in Charts 2 and 7—11 may be considered forthwith.

The diets used in this series of experiments were similar in composition to those employed by Osborne and Mendel [1911].

Purified protein	18 %	Agar	5 %
Butter-fat	20 %	Starch	27 %
"Protein-free milk"	30 %		

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat − tumour g	Change in weight of rat g	Change in weight of rat per 1 g. tumour
♂	•	●	●	●	●	Tumour very necrotic and ulcerated.	29·0	83·0	−9·0	
♀	•	●	●	●	●	ditto	19·0	77·0	+15·0	
♀	•	●	●	●	–	ditto	9·0	49·0	−11·0	
♀	•	•	●	●	●	Tumour necrotic	4·0	86·0	+26·0	
						Average	15·2	–	+5·2	+0·34

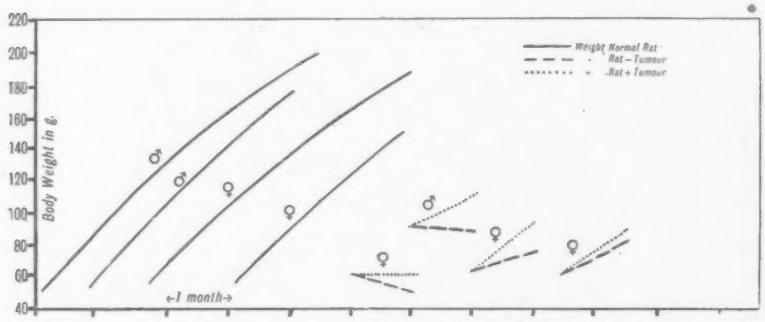


Chart 8. Tumour development, and growth curves of normal and tumour-bearing rats upon Lactalbumin diet. Tumour *A*/30♂. Diet contains 18 % Lactalbumin.

The following proteins were investigated: caseinogen, lactalbumin, ovovitellin, edestin, gliadin, and zein.

The influence of these diets upon tumour growth may be summarised in the following table:

TABLE 2.

Protein in diet	Growth of normal animal on diet	Average weight of tumour, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. tumour in four weeks
Caseinogen	normal	21·7	− 1·8	−0·08
Edestin	normal	15·5	+ 4·2	+0·27
Lactalbumin	normal	15·2	+ 5·2	+0·34
Ovovitellin	normal	17·0	+ 5·7	+0·34
Gliadin	cessation of growth and maintenance at constant weight	21·1	− 9·9	−0·47
Zein	rapid decline in body-weight ...	17·5	−21·2	−1·20

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of host g.	Change in weight of rat per 1 g. tumour
♂						Tumour ulcerated and necrotic.	20·0	72·0	+11·0	
♀						Tumour extensively necrotic.	22·0	78·0	+9·0	
♀						Tumour very necrotic	16·0	85·0	−5·0	
♂						Slight central necrosis	10·0	90·0	+8·0	
						Average	17·0	—	+5·7	+0·34

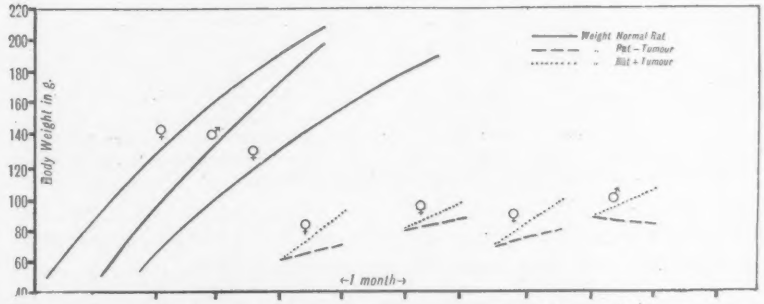


Chart 9. Tumour development, and growth curves of normal and tumour-bearing rats upon Ovo-vitellin diet. Tumour *A*/30*d*. Diet contains 18 % Ovo-vitellin.

For comparative purposes these results are valuable in that they indicate that tumour growth may be uninfluenced by dietary restrictions which

adversely affect the growth of the host. Individually, however, the results are unsatisfactory because of the irregularity in the changes in the body-weight of the rats on the first four dietaries. Thus there is no apparent explanation of the decrease of body-weight shown by the tumour-bearing rats fed upon the caseinogen diet. The only suggestion which can be advanced to explain this is that the *A* tumour was used throughout this experiment. As has already been mentioned this tumour tends to show an early

Rat	Days	Remarks	Weight of tumour g	Weight of rat – Tumour g	Change in weight of rat g	Change in weight of host per 1 g tumour
♀		Tumour extensively necrotic.	42·0	48·0	−10·0	
♀		Tumour very necrotic and ulcerated.	28·0	32·0	−19·0	
♀		Tumour firm, slight central necrosis	10·0	60·0	−12·0	
♀		Small fibrous growth	4·5	61·5	+1·5	
♀		Tumour retrogressed	–	–	–	
		Average	21·1	–	−9·9	−0·47

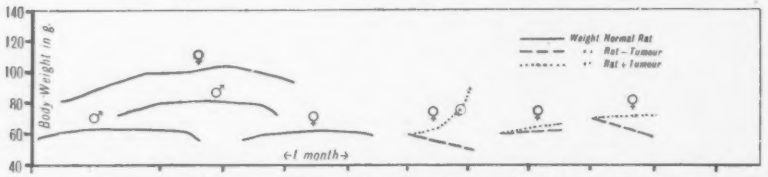


Chart 10. Tumour development and growth curves of normal and tumour-bearing rats upon Gliadin diet. Tumour *A*/30*d*. Diet contains 18 % Gliadin.

and extensive central necrosis, so that the possible deleterious influence upon the nutrition of the host caused by the absorption of the products arising from the degenerating tissue must be considered. Whatever may be the explanation of this point, it does not modify the importance of the observation that tumour growth may be practically normal in hosts whose growth is inhibited by the nature of their diet. The results obtained upon the gliadin

ration are of interest in view of the large amount of work which has been done upon the nutritive value of that protein.

Osborne and Mendel [1914, 1; 1916, 2] found that gliadin was adequate to supply the amino-acid requirements for maintenance in the rat, but that the addition of some 2 % of the missing unit, lysine, was necessary before the requirements for growth were satisfied.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat − tumour g	Change in weight of rat g	Change in weight of rat per 1 g tumour
♂						Tumour very necrotic, animal extremely emaciated	21·0	29·0	−36·0	
♀						ditto	14·0	37·0	−11·0	
♂						ditto	15·0	38·0	−18·0	
♀						Animal emaciated Tumour shows slight central necrosis	10·0	52·0	−20·0	
♀						—	—	—	—	
♀						Tumour retrogressed	—	—	—	
						Average	17·5	—	−21·2	−1·20

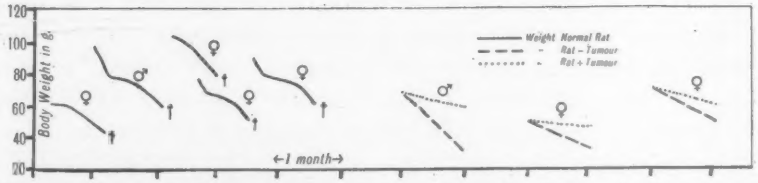


Chart 11. Tumour development, and growth curves of normal and tumour-bearing rats upon Zein diet. Tumour *A*/30*d*. Diet contains 18 % Zein.

It was the earlier results of Osborne and Mendel on this subject [1912, 1] that stimulated the investigations of Sweet, Corson-White and Saxon [1913], to which reference has already been made. The view which regards lysine as of great importance in growth receives support from the work of Buckner, Nollau and Kastle [1916] who worked upon the growth of chickens, and also from similar studies of Osborne and Mendel [1916, 4]. Other workers however do not attach as great an importance to the rôle of this amino-acid in nutrition. Thus McCollum, Simmonds and Pitz [1917] have shown that the

addition of gelatin, which possesses a high lysine content, does not improve the nutritive value of the lysine-poor proteins of the maize kernel, whilst Geiling [1917] does not regard lysine as necessary for the maintenance of the adult mouse. The true value of lysine in the nutrition of the organism is therefore at present uncertain.

Equal interest is to be attached to the results illustrated in Chart 11, where the influence of the well-known amino-acid deficiencies of the protein zein upon normal and tumour growth are indicated. The low nutritive value of this protein was shown by Willcocks and Hopkins [1906] to be largely due to the absence of tryptophan from its molecule. Their work has been repeatedly confirmed, and the serious nature of a tryptophan deficiency is now universally admitted. As is to be seen from Chart 11, young rats rapidly decline in body-weight and die upon a zein ration such as was used in this experiment.

It was, therefore, somewhat unexpected when no inconsiderable amount of tumour development was observed in rats fed upon the same ration. In the light of the results of some later experiments with zein dietaries, attention must be given to one of the criticisms which have been employed in deprecating the use of "protein-free milk," namely that the traces of milk protein which that product contains may possibly supply traces of the missing amino-acids, which may be of welcome assistance to the animal in its endeavour to overcome the serious deficiencies of its diet. Whether this will to some extent explain the result of this feeding experiment is uncertain, but it is apparent from a consideration of the experiment about to be described that a tryptophan deficiency, in the absence of any uncertain factor such as "protein-free milk," may cause retardation of tumour growth. Chart 12 illustrates the results which were obtained with a ration composed as given below:

Zein	18 %	Butter-fat	10 %
Lactose	30 %	Salt mixture	5 %
Starch	40 %		

Alcohol extract of 20 g. milk powder.

The summarised results of the experiments in this series are given in Table 3.

TABLE 3.

Diet	Average weight of tumour, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. tumour in four weeks
18 % caseinogen	12·0	+19·9	+1·66
18 % zein	4·1	−21·1	−5·15
18 % zein + 0·5 % tryptophan ...	9·2	− 9·8	−1·06

As will be seen from the third series in this table, the influence of the addition of the missing amino-acid was investigated (Chart 13). In both the normal and tumour-bearing rats this addition markedly improved the nutritive value of the ration. Upon the zein diet there was a distinct retardation, but not a total inhibition, of tumour growth which was accompanied by a very rapid fall in body-weight of the host.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of rat g.	Change in weight of host per 1 g. tumour
♀	•	•	•	•	•	Tumour fibrous, but well vascularised	6·0	74·0	−13·0	
♂	•	•	•	•	—	Animal very thin.	5·0	53·0	−27·0	
♀	•	•	•	•	•	Tumour slow growing. Animal very thin.	2·5	58·5	−24·5	
♀	•	•	•	•	—	—	3·0	45·0	−20·0	
♂	•	•	•	•	—	Tumour retrogressed	—	—	—	
♂	•	•	—	—	—	ditto	—	—	—	
						Average	4·1	—	−21·1	−5·15

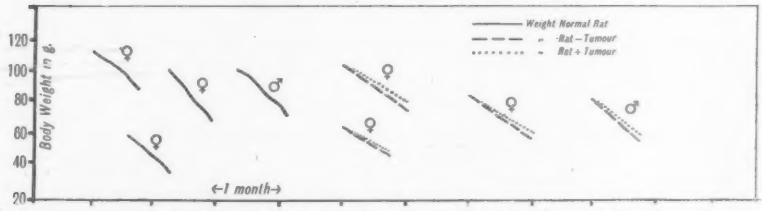


Chart 12. Tumour development, and growth curves of normal and tumour-bearing rats upon Zein diet. Tumour *S*/116*b*. Diet: Zein, 18 %, Crude lactose, 30 %, Starch, 40 %, Butter-fat, 10 %, Salt mixture, 5 % + Alcoholic extract 20 g. dried milk.

The addition of tryptophan to the extent of 0·5 % of the diet both assisted the growth of the tumour and lessened the sacrifice of the tissues of the host. It should be remarked that the consumption of the dietaries was satisfactory, except that of the zein ration during the fourth week, which declined considerably.

The low nutritive value possessed by gelatin has been recognised for many years. It has been shown that gelatin cannot serve to build up new tissue; whilst Kauffmann [1905] demonstrated that when the amino-acids

tyrosine, cystine and tryptophan are added to gelatin, and the deficiencies of the latter thereby to a large extent made good, nitrogen equilibrium can be established.

His work has received confirmation from that of Rona and Müller [1906] and Abderhalden [1912]. More recently the subject has been carefully investigated by Totani [1916], who found that the addition of tryptophan to gelatin greatly raised its nutritive value. The effect of adding the missing tyrosine was hardly appreciable; so that the author was led to suggest that the animal organism may have the power of synthesising the benzene nucleus.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♂	●	●	●	●	●	Tumour slightly necrotic. Animal thin.	14·0	78·0	−9·0	
♀	●	●	●	●	●	ditto	14·0	69·0	−12·0	
♀	●	●	●	●	●	ditto	7·0	92·0	−8·0	
♂	●	●	●	●	●	Tumour firm and fibrous. Animal thin.	2·0	90·0	−10·0	
♀	●	●	●	—	—	Tumour retrogressed	—	—	—	
						Average	9·2	—	−9·8	−1·06

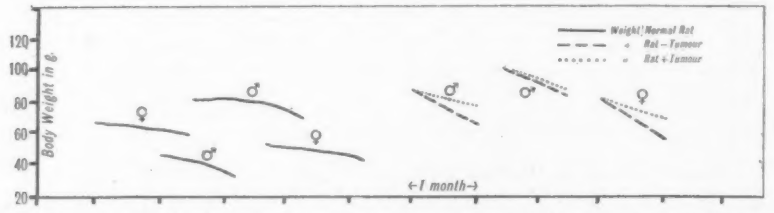


Chart 13. Tumour development, and growth curves of normal and tumour-bearing rats upon Zein diet (Chart 12) + 0·5 % tryptophan. Tumour *S*/116*b*.

The investigation of the influence of the amino-acid deficiencies of gelatin upon tumour growth, which are described in this paper, was carried out before the appearance of Totani's communication, so that his observation upon the more complete assimilation of gelatin administered in the hydrolysed

form was not applied. The gelatin was included in the diets in these experiments in the powder form.

The average results obtained in the four batches of experimental animals are tabulated in Table 4, which has been compiled from the data contained in Charts 14, 15 and 16.

Rat	Days 12	19	26	33	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♂					Rat very emaciated. Tumour firm, well vascularised and actively growing.	12·0	60·0	−40·0	
♂					ditto	10·5	74·5	−29·5	
♂					ditto	5·0	82·0	−28·0	
♀					ditto	4·0	73·0	−27·0	
♀					Tumour fibrous and slow growing. Rat very thin	2·5	65·5	−22·5	
					Average	6·8	—	−29·4	−4·3

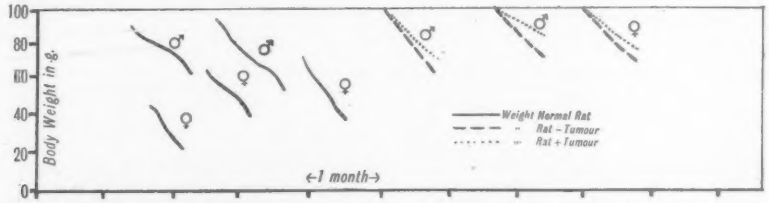


Chart 14. Tumour development, and growth curves of normal and tumour-bearing rats upon Gelatin diet. Tumour *S*/118*a*. Diet: Gelatin, 18 %, Starch, 52 %, Lard, 20 %, Agar, 5 %, Salt mixture, 5 % + Alcoholic extract 20 g. dried milk.

TABLE 4.

Diet	Average weight of tumour, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. of tumour
18 % caseinogen	14·2	+19·0	+1·34
18 % gelatin	6·8	−29·4	−4·30
18 % gelatin + 0·5 % tryptophan	12·7	−19·3	−1·52
18 % gelatin + 0·5 % tryptophan + 1 % tyrosine	13·2	−16·5	−1·25

The influence of these dietaries upon the growth of the normal rats supports the work of Totani, in that the addition of tryptophan greatly raises the nutritive value of the gelatin diet, whereas the further addition of tyrosine has little if any such effect.

The retarding influence upon tumour growth which is exerted by the amino-acid deficiencies of the gelatin ration is well marked. As in other similar cases this is accompanied by a severe drop in the body-weight of the host.

Rat	Days 12	19	26	33	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of rat g.	Change in weight of host per 1 g. tumour
♀					Rat thin Tumour shows slight central necrosis	18·5	71·5	−20·5	
♂					ditto	15·0	85·0	−28·0	
♂					ditto	14·0	94·0	−16·0	
♂					ditto	11·0	65·0	−25·0	
♀						5·0	83·0	−7·0	
					Average	12·7	—	−19·3	−1·52

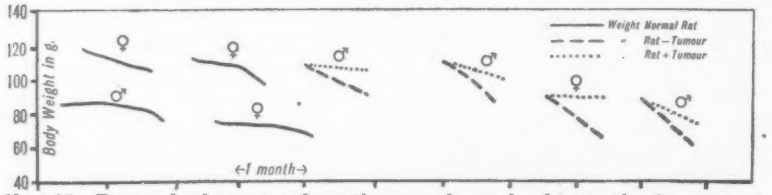


Chart 15. Tumour development, and growth curves of normal and tumour-bearing rats upon Gelatin diet, described in Chart 14, + 0·5 % tryptophan. Tumour *S*/118*a*.

When tryptophan has been added to the diet, tumour growth becomes normal, but there is a slight loss of weight upon the part of the host. The further addition of the missing tyrosine cannot be said appreciably to influence either tumour growth, or the sacrifice of the tissues of the animal.

The gelatin dietary to which the missing tryptophan and tyrosine have been added possesses a much lower nutritive value than the "normal"

caseinogen ration. This is probably to a large extent due to the defective assimilation of the solid gelatin, which has been observed by Totani. This imperfect utilisation does not apparently adversely affect tumour growth when tryptophan is added, but is reflected in the lowered nutrition of the host.

Rat	Days 12	19	26	33	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♂	•	•	●	●	Tumour shows slight central necrosis	17·0	79·0	−22·0	
♂	•	•	●	●	ditto	16·0	83·0	−17·0	
♀	•	•	●	●	ditto	11·0	75·0	−12·0	
♀	•	•	●	–	Animal died, and partially devoured 20.1.17	9·0	75·0	−15·0	
♀	•	•	•	•	Tumour fibrous, with small blood supply	–	–	–	
					Average	13·2	–	−16·5	−1·25

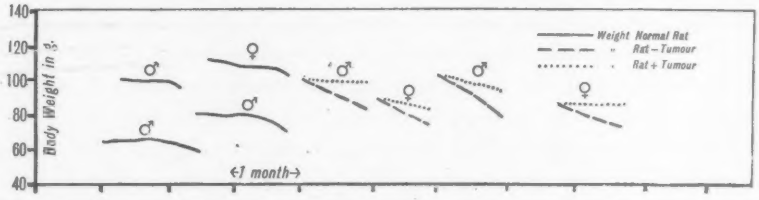


Chart 16. Tumour development, and growth curves of normal and tumour-bearing rats upon Gelatin diet + 0·5 % tryptophan + 2·0 % tyrosine. Tumour *S*/118*a*.

The effect upon normal and tumour growth of replacing the protein of a diet by amino-acid mixtures.

It is mainly due to the extensive researches of Abderhalden that it is now proved that an animal can be maintained, and even show an increase of body-weight, when it is fed upon a diet in which protein has been entirely replaced by an amino-acid mixture prepared from a completely hydrolysed protein. Of outstanding importance in this respect are the classical experi-

ments made by him, in which he accomplished the successful nutrition of dogs over long periods of time upon such diets [1913, 2; 1915].

He also found the same amino-acid preparation adequate to supply the nitrogen requirements of rats.

Abderhalden has extended his researches so as to ascertain the effect which the removal of certain amino-acids from his protein digest has upon the nutrition of the animal [1915].

By removing tryptophan and tyrosine the nutritive value of the hydrolysate was greatly lowered, becoming totally insufficient to maintain the body-weight of the animals. Restoration of the missing amino-acids was immediately effective in correcting this deficiency.

The removal of lysine was followed by a negative nitrogen balance, as was also the administration of a preparation of hydrolysed gliadin. The addition of the missing lysine did not in these two cases completely restore the food value of the rations. Other experiments dealing with the relative importance of histidine, arginine, and cystine gave somewhat indefinite results; although the evidence in the last case tended to show that cystine is an indispensable component of the protein molecule. Equally interesting results have been obtained by Hopkins and his co-workers in this country [Hopkins, 1916].

Ackroyd and Hopkins [1916] have again confirmed the absolute indispensability of tryptophan, and have also obtained most interesting and important results bearing upon the physiological value of the amino-acids arginine and histidine. They have presented evidence which indicates that these two substances are interconvertible in the animal organism and that an adequate supply of at least one of them in the diet is necessary for satisfactory nutrition. From their results it would also appear that these amino-acids can furnish the starting point of purine synthesis in the animal body.

Geiling [1917] has recently carried out an investigation the results of which have once again demonstrated the importance of the so-called diamino-acids in the processes of nutrition and growth.

This worker regards tryptophan and cystine as indispensable dietary units, and he adds confirmation of the relationship between arginine and histidine, described by Ackroyd and Hopkins. He does not regard lysine as of special importance.

Attempts have been made to effect the nutrition of animals upon diets in which protein has been replaced by artificial mixtures of amino-acids in

the pure state. Abderhalden [1912] encountered great experimental difficulties in attempting this, but was successful in maintaining a dog for eight days at constant body-weight upon a diet containing all the common amino-acids excepting oxyproline. The nutrition of the animal was far less satisfactory than when "erepton"—a completely hydrolysed meat-protein—was employed.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat—Tumour g.	Change in weight of rat g.	Change in weight of host per 1 g. tumour
♂						Tumour extensively necrosed and ulcerated. Animal thin.	32·0	134·0	+20·0	
♂						ditto.	27·0	99·0	−18·0	
♂						Tumour very necrotic	12·0	114·0	+14·0	
♀						Tumour small and fibrous	–	–	–	
						Average	23·6	–	+5·3	+0·22

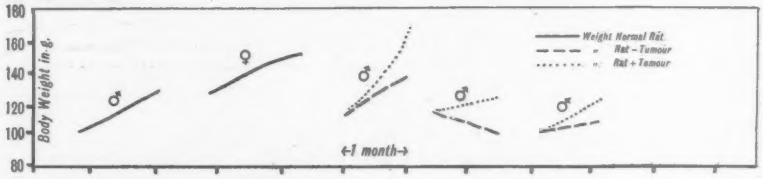


Chart 17. Tumour development, and growth curves of normal and tumour-bearing rats upon "Erepton" diet. Tumour *S*/121*d*.

Osborne and Mendel [1916, 2] record a failure to maintain rats upon a non-protein diet containing tryptophan, cystine, histidine, tyrosine, phenylalanine, proline and ammonium salts. Mitchell [1916] working with mice, has to some extent succeeded in surmounting the great difficulties which impede experimentation along these lines. His results support the indispensability of tryptophan, and indicate that the benzene nucleus may be omitted from the diet without adversely affecting nutrition.

It will be seen from a summary of these results that there is general agreement in regarding tryptophan, cystine and arginine or histidine as indispensable dietary components, but that opinion is divided regarding the value of lysine and the amino-acids containing the benzene nucleus.

The application of these results to the determination of the influence of individual amino-acids upon tumour growth was carried out in a similar manner to that employed in the study of the nutrition of the normal animal by Abderhalden and by Hopkins. Two amino-acid mixtures were prepared

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat−tumour g.	Change in weight of host g.	Change in weight of host per 1 g. tumour
♂	•	■	●	●	●	Tumour somewhat firm and fibrous. Animal very emaciated	8·5	67·5	−35·5	
♀	•	•	●	●	●	ditto	8·0	56·0	−18·0	
♂	•	●	●	●	–	Tumour well vascularised, firm and slow growing. Animal very emaciated	8·0	67·0	−33·0	
♂	•	•	•	●	–	ditto	3·5	46·5	−15·5	
♀	•	●	●	–	–	Killed and devoured 28. 12. 16	–	–	–	
						Average	7·0	–	−25·5	−3·6

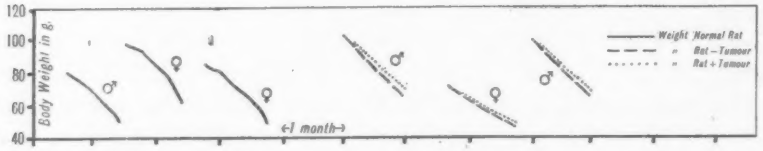


Chart 18. Tumour development, and growth curves of normal and tumour-bearing rats upon acid-hydrolysed meat-protein diet. Tumour *S*/117*c*.

from hydrolysed muscle protein, the one containing all the amino-acids excepting tryptophan, whilst from the other all the amino-acids which are precipitated by phosphotungstic acid had been removed. A commercial sample of completely hydrolysed meat-protein ("erepton") containing tryptophan, and prepared according to the method of Abderhalden, served as a control to these preparations.

The general composition of the rations employed is given below.

"Erepton" diet		Hydrolysed meat protein diet		"Monamino-acid fraction" diet	
"Erepton" (12·5 % N)	20 %	Acid-hydrolysed muscle preparation (7 % N)	40 %	"Monamino-acid fraction" preparation (5 % N)	50 %
Starch	40	Starch	20	Starch	25
Sucrose	15	Sucrose	15	Salt mixture ...	5
Salt mixture	5	Salt mixture	5	Agar	5
Agar	5	Agar	5	Butter-fat	15 +
Butter-fat	15 +	Butter-fat	15 +	Alcoholic extract of dried milk	20 g.
Alcoholic extract of dried milk	20 g.	Alcoholic extract of dried milk	20 g.		

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour necrotic and ulcerated. Animal thin.	19·0	55·0	−23·0	
♀						Tumour firm Animal thin.	16·0	62·0	−28·0	
♀						ditto	16·0	72·0	−25·0	
♀					—	ditto	10·0	57·0	−14·0	
♂					—	ditto	9·0	80·0	−10·0	
♀						ditto	3·0	77·0	−7·0	
						Average	12·0	—	−17·8	−1·52

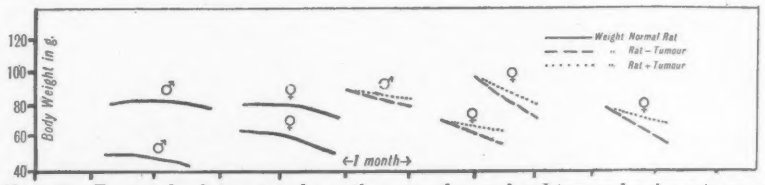


Chart 19. Tumour development, and growth curves of normal and tumour-bearing rats upon acid-hydrolysed meat-protein diet + 0·5 % tryptophan. Tumour *S*/117c.

Amino-acid additions were made, where indicated, in the following amounts:

Tryptophan	0·5	% of total diet
Cystine	0·5	„ „
Lysine dihydrochloride (in neutral solution)	2·0	„ „
Arginine nitrate „ „	2·0	„ „
Histidine hydrochloride „ „	1·5	„ „
Tyrosine	2·0	„ „

Throughout the course of this series of experiments some difficulty was encountered in getting the rats to consume adequate amounts of the diets. This was particularly marked in the case of rations to which had been added the various pure amino-acids. Many attempts were made to render the diets more palatable without materially altering their composition but without much success.

The average results of this series of experiments are summarised in Table 5.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour firm. Animal very emaciated	15·0	53·0	−50·0	
♀						Slight central necrosis of tumour	13·0	48·0	−48·0	
♀						Tumour retrogressing	—	—	—	
						Average	14·0	—	−49·0	−3·5

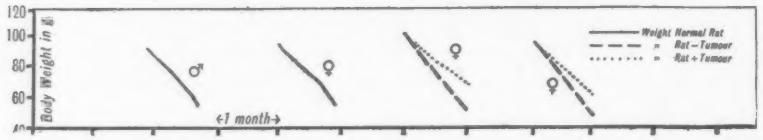


Chart 20. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet. Tumour *S*/120*d*.

TABLE 5.

Diet	Average weight of tumour, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. tumour
"Erepton"	23·6	+ 5·3	+0·22
Acid-hydrolysed protein	7·0	−25·5	−3·50
Acid-hydrolysed protein + 0·5 % tryptophan	12·0	−17·8	−1·52
"Monamino-acid fraction" + lysine, arginine, histidine, cystine and tryptophan	17·0	+1·50	+0·89
"Monamino-acid fraction" + lysine, arginine, histidine, and tryptophan	16·3	−10·3	−0·63
"Monamino-acid fraction" + lysine, arginine and histidine	15·0	−33·0	−2·20
"Monamino-acid fraction" + arginine and histidine ...	21·0	−39·0	−1·90
"Monamino-acid fraction" + tryptophan	19·5	−29·0	−1·49
"Monamino-acid fraction"	14·0	−49·0	−3·50

These results are illustrated more fully in Charts 17 to 25. It is unfortunate that the great labour involved in the preparation of the materials for this work should have necessitated a limitation of the number of experimental animals. This fact naturally greatly reduces the value of the average figures given in the preceding table, but it does not lessen the importance of observations that tumour growth was possible upon certain diets. The normal rats fed upon the "erepton" diet exhibited a sub-normal rate of growth, which may be attributable in part to the low food consumption of the animals. Tumour growth was quite normal in hosts fed upon the same ration, and was not accompanied by a loss of tissue upon the part of the animal (Chart 17).

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour somewhat necrotic and ulcerated	22	63	−39	
♂						Tumour firm	17	73	−19	
						Average	19·5	—	−29	−1·49

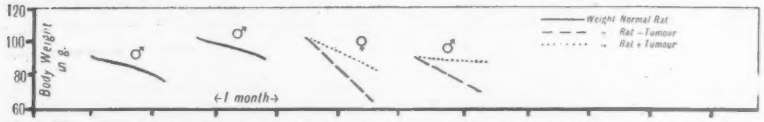


Chart 21. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet + 0·5 % tryptophan. Tumour *S*/120*d*.

The diet prepared with the acid-hydrolysed meat-protein plus tryptophan should on theoretical grounds possess a nutritive value almost equal to that of "erepton." As a matter of fact it was found to possess a somewhat lower food value for normal rats, whilst tumour growth appeared somewhat retarded (Chart 19).

The adverse effects upon the nutrition of the animal which follow the withdrawal of tryptophan from the diet are illustrated in Chart 18. Normal animals quickly declined in weight and succumbed on this inadequate diet, whilst the tumour rats showed an appreciable inhibition of tumour growth, accompanied by a rapid fall in the body-weight of the host.

The low nutritive value possessed by an amino-acid mixture which is deficient in the so-called diamino-acids is illustrated in Chart 20. This ration was totally inadequate for the nutrition of normal rats, but the same restrictions imposed upon the tumour-bearing animals brought about comparatively little retardation of tumour growth.

As has already been remarked, it is not possible to place much reliance in the data given in Table 5, as in many cases they represent the average figures for a very small number of subjects.

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Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of Rat − tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour shows slight central necrosis Animal very thin	30	69	−49	
♀						ditto	12	75	−29	
						Average	21	—	−39	−1·9

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♀						Tumour firm. Animal very thin.	15	75	−32	
♂						ditto	15	72	−34	
						Average	15		−33	−2·2

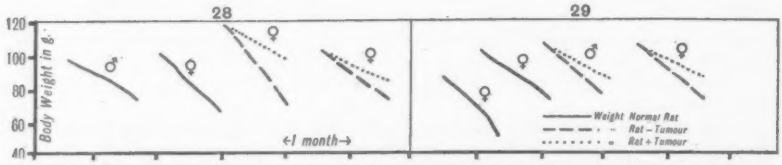

Chart 22. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet, + 2 % arginine nitrate + 1·5 % histidine hydrochloride. Tumour *S*/120*d*.

Chart 23. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet + 2 % arginine nitrate + 1·5 % histidine hydrochloride + 2 % lysine dihydrochloride. Tumour *S*/120*d*.

From the other Charts, 21—25, may be seen the influence exerted upon the nutrition of the experimental animals by additions of the various missing amino-acids.

The outstanding feature of this series of experiments is the demonstration

of the ability of the rat sarcoma to grow with comparatively little retardation in a host subjected to most drastic dietary restrictions.

Since it is apparent that the cells of these tumours can obtain considerable supplies of food units necessary for growth, when these are not present in the diet of the host, it becomes of importance to determine from what sources they are procured by the tumour.

30

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of host per 1 g. tumour
♂	•	•	●	●	●	Tumour much necrosed and ulcerated	24	96	−14	
♀	•	•	•	●	●	Tumour firm, shows slight central necrosis	14	96	+1	
♀	•	•	●	●	●	ditto	11	90	−18	
						Average	16·3	—	−10·3	−0·63

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♀	•	•	●	●	●	Tumour firm, slight central necrosis	20	90	+1	
♀	•	•	●	●	●	ditto	14	99	+2	
						Average	17	—	+1·5	+0·89

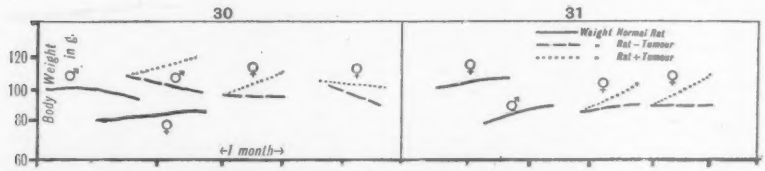

Chart 24. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet, + 2 % arginine nitrate + 1·5 % histidine hydrochloride + 2 % lysine + 0·5 % tryptophan. Tumour *S*/120*d*.

Chart 25. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet, + 2 % arginine nitrate + 1·5 % histidine hydrochloride + 2 % lysine dihydrochloride + 0·5 % tyrosine + 0·5 % cystine + 0·5 % tryptophan. Tumour *S*/120*d*.

Tumour cells require a comparatively large supply of the diamino-acids for their growth; since it has been shown by the analysis of a large number of tumour proteins that they contain a high percentage of these substances [Drummond, 1916, 3]. There is no evidence which indicates that tumour

cells differ from normal tissue cells in possessing the power to synthesise important cell units, such as the diamino-acids.

The question therefore arose as to whether the composition of the tumour proteins was in any way influenced by restrictions in the amino-acid intake of the host. Accordingly a number of the tumours grown in hosts fed upon certain of the diets employed in the last series of experiments were submitted to analysis in order to determine the amino-acid distribution of their proteins.

The preparation of the tumour material for analysis and the details of the latter were as described in a previous communication [Drummond, 1916, 3]. The partition of the various units constituting the diamino-acid fraction was not studied, in view of the fact that there appears to be some doubt as to the accuracy of the methods usually employed for that purpose.

A number of analyses were also carried out upon the proteins of the tissues of the host. Unfortunately the small amount of material furnished by individual organs prevented separate analyses being made of these. A representative sample of the tissues of the animal was therefore analysed. To ensure uniformity, the sample was composed, in each case, of the heart, lungs, liver, spleen, one kidney, equal sized strips of skeletal muscle, and one testicle or the ovary.

TABLE 6.

Partition of nitrogen in proteins of rat sarcoma S, and in the proteins of the tissues of hosts bearing these tumours.

Nature of diet of tumour-bearing rat	Protein analysed	No. of tissue	% N in dry tissue	% Amide N	% Humin N	Monamino-acid filtrate: % Monamino-acid N	Monamino-acid filtrate: % Nonamino-acid N	% Diamino-acid N	Total % N recovered
Normal diet	Organs	1	12·80	6·27	4·61	50·56	6·53	32·23	100·20
	Tumour	2	13·83	5·25	3·16	50·80	5·40	34·42	99·03
"Erepton" diet	Organs	3	12·62	5·51	5·66	55·93	4·77	30·97	100·64
	Tumour	4	13·55	4·42	3·59	51·77	6·73	32·42	98·93
Hydrolysed meat protein diet (tryptophan absent)	Organs	5	12·78	4·83	4·87	51·90	4·60	32·20	98·40
	Tumour	6	13·81	4·69	4·25	52·68	6·12	31·72	99·36
Monamino-acid diet	Organs	7	12·37	4·93	4·73	52·60	3·95	32·72	98·96
	Tumour	8	13·84	5·02	3·98	52·40	6·50	32·00	99·10

In the animals which had suffered a severe loss of body-weight upon certain of the diets, the post-mortem examination showed great wasting of the skeletal muscles. Blood examination of such animals carried out some days before death frequently revealed a state of anaemia. The number of animals under investigation was unfortunately too small to permit of a comparison being made of the weights of the various organs in these cases with the standard

data given by Donaldson [1915]. No apparent wasting was noticeable upon examination of the heart, spleen or kidneys, but the liver in some cases appeared smaller than normal.

Table 6 contains the results obtained from the examination of the tumour and tissue proteins referred to above. A study of this table indicates, as far as it goes, that the composition of both the tumour proteins and the proteins of the tissues of the host is unaffected as a result of deficiencies in the amino-acid content of the diets.

This chemical evidence is supported by the histological examination of the tumours and organs removed from the tumour-bearing rats. No appreciable differences could be detected between the microscopical appearance of sections of the tumours grown in animals fed upon the diets showing serious amino-acid deficiencies, and those removed from normally fed animals. Even the tumours removed from the rats fed upon the amino-acid diet deficient in histidine, arginine, cystine, lysine and tryptophan presented an apparently normal microscopical appearance.

The influence of the accessory growth factors "fat-soluble A" and "water-soluble B," upon tumour growth in rats.

The importance of these two unidentified dietary factors in the nutrition of the growing animal has received attention in the introduction to this paper. To investigate the possible influence which they might exert upon tumour growth the following four rations were prepared. Diet *A* contained both factors, and being in other respects adequate, may be considered a "complete" diet. Diet *B* lacked the "fat-soluble *A*" factor, but in other respects possessed a similar composition to *A*. Diet *C* was deficient only in the "water-soluble *B*" substance, whilst Diet *D* contained neither accessory factor.

The composition of the dietaries was as given below.

	A	*B*	*C*	*D*
Caseinogen	18	18	18	18
Starch	48	48	54	54
Agar	3	3	3	3
Salt mixture	5	5	5	5
"Yeast preparation"	6	6	—	—
Butter-fat	20	—	20	—
Lard	—	20	—	20

The results which were obtained are recorded in Charts 2, 26, 27 and 28 and are summarised in Table 7. Charts 29–31 refer to a similar experiment dealing with the fat-soluble *A*.

TABLE 7.

Diet	Deficiency in diet	Average weight of tumour, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. tumour
A	Nil	16·6	+42·0	+2·5
B	Fat-soluble *A*	16·3	+12·9	+0·9
C	Water-soluble *B*	13·5	− 3·5	−0·3
D	Fat-soluble *A* and water-soluble *B*	12·7	− 5·0	−0·4

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Solid growth	10·5	87·5	−3·5	
♀						Tumour very necrotic, ulcerated 21. 4. 17.	22·5	71·5	−10·5	
♀						Tumour small and fibrous	2·0	90·0	−1·0	
♀						Tumour centrally necrotic	24·0	71·0	−4·0	
♂					–	Died 19. 4. 17	8·5	81·5	+1·5	
						Average	13·5	–	−3·5	−0·28

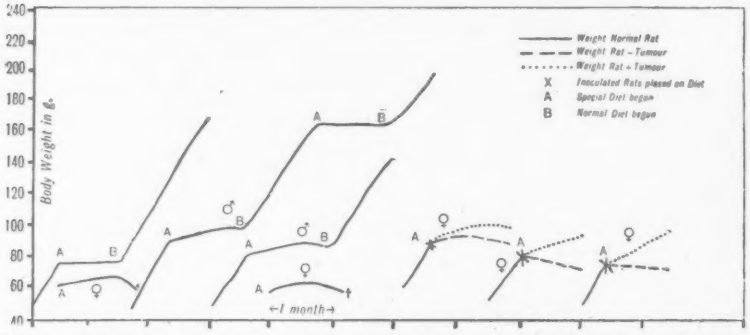

Chart 26. Tumour growth, and growth curves of normal and tumour-bearing rats upon diet deficient in "water-soluble *B*." Tumour *S*/120*d*. Diet similar to that employed for rats in Chart 2, except that "Yeast preparation" was omitted.

From these average figures, as well as from a study of the charts, it may be seen that no retardation of tumour growth followed a deficiency of the fat-soluble *A*, whereas the absence of the water-soluble *B* did cause a certain amount of inhibition. Even in the case of diet *D*, where both factors were

absent, the inhibition of tumour growth is only of the same order as that brought about by the deficiency of the water-soluble factor alone. The explanation of these results may be assisted by a study of the growth curves of the normal animals fed upon these rations. A deficiency in the supply of the fat-soluble factor in the diet of a growing rat is not always followed by

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat – tumour g	Change in weight of rat g	Change in weight of rat per 1 g. tumour
♂						Tumour firm	7·0	114·0	+21·0	
♀						Large tumour extensively necrotic, small nodule solid	24·5	74·5	+2·5	
♂						Tumour firm	10·0	102·0	+2·0	
♂						Slightly necrotic at centre	38·0	128·2	+27·2	
♂						Hard fibrous growth	0·8	100·0	+12·0	
						Average	16·1	—	+12·9	+0·80

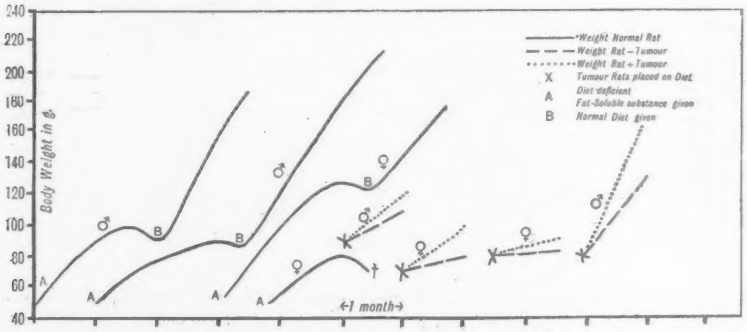

Chart 27. Tumour development, and growth curves of normal and tumour-bearing rats upon diet deficient in "fat-soluble *A*." Tumour *S*/120*d*. Diet similar to that employed in Chart 2, but butter-fat was replaced by lard.

an immediate cessation of growth. More usually, growth continues at a more or less subnormal rate for a period dependent upon the health and age of the animal. An animal which is well nourished at the outset of such an experiment may show such growth for several weeks; sooner or later, however, a cessation of growth will ensue which will be followed by a rapid

decline and death. Only a prompt addition of the missing factor to the diet will prevent this decline and restore health and growth.

These facts have led to the suggestion being advanced that the animal organism can mobilise existing stores of this dietary factor when a deficiency

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour ulcerated and very necrotic. Rat very thin and anaemic	23·0	81·0	−13·0	
♂						Firm growth	6·5	84·5	+0·5	
♀						Firm growth	8·5	92·5	−9·5	
♀						Somewhat fibrous tumour	3·0	98·0	+2·0	
♂					—	Tumour retrogressed	—	—	—	
						Average	10·2	—	−5·0	−0·49

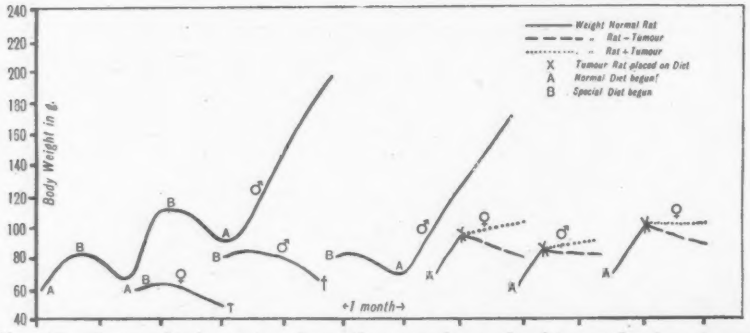

Chart 28. Tumour development, and growth curves of normal and tumour-bearing rats upon diet deficient in "water-soluble *B*," and "fat-soluble *A*." Tumour *S*/120*d*. Diet composed as described under Chart 2, but "Yeast preparation" omitted, and butter-fat replaced by lard.

occurs in its diet [Halliburton and Drummond, 1917]. Since there is evidence which indicates that the animal is able to grow for some weeks upon a diet deficient in the fat-soluble *A*, by making use of its own store of that substance, it is not surprising to find normal tumour growth in hosts placed under the same nutritive condition. Whether a deficiency in this factor

would exert a retarding influence upon tumour development after the available reserves had been exhausted is uncertain.

Animals are much less able to withstand the consequences following the withdrawal of the water-soluble *B* from the diet. As is seen from the curves of the normal rats, the withdrawal of this substance is practically immediate

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour extensively necrotic at centre	32·0	57·0	−13·0	
♂						Slight central necrosis	17·0	59·0	−2·0	
♀						—	7·0	103·0	+3·0	
♂						Ulcerated. considerable necrosis at centre of tumour	30·0	82·0	+6·0	
♀						ditto	40·0	57·0	−19·0	
						Average	25·2	—	−5·0	−0·20

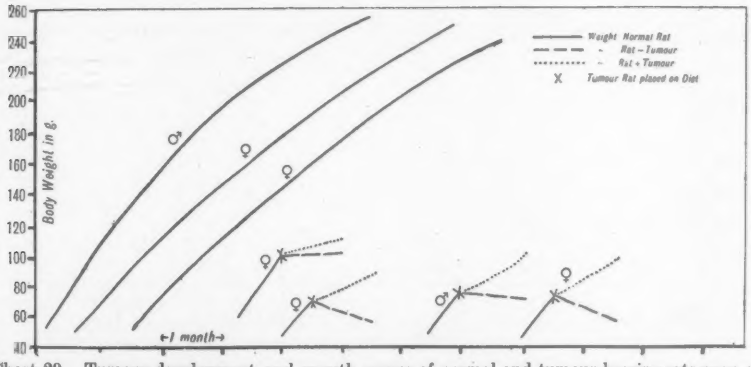

Chart 29. Tumour development, and growth curves of normal and tumour-bearing rats upon a complete artificial dietary. Tumour *S*/117*d*. Diet: Dried milk powder, 50 %, Starch, 40 %, Lard, 10 %.

in causing a cessation of growth. Then follows a period of maintenance or slow decrease in body-weight which eventually ends with a sharp decline and death. What is the state of the animal with regard to this dietary factor during this period is undefined. Drummond [1917] has recently shown that

the properties of the water-soluble growth factor and those of the so-called "beri-beri vitamine" are so closely similar as to render it very probable that the two substances are identical. If this is so certain facts known regarding the latter body may help to throw light on the question. Thus it is well known that the onset of the typical symptoms of the condition known as

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour shows slight central necrosis	21	43	−16	
♀						Firm tumour	20	74	−10	
♂						Firm, slow growing	5·5	78·5	−3·5	
♂						Firm tumour	10	70	−15	
♀						Centrally necrotic and ulcerated	40	39	−28	
						Average	19·3	—	−14·5	−0·75

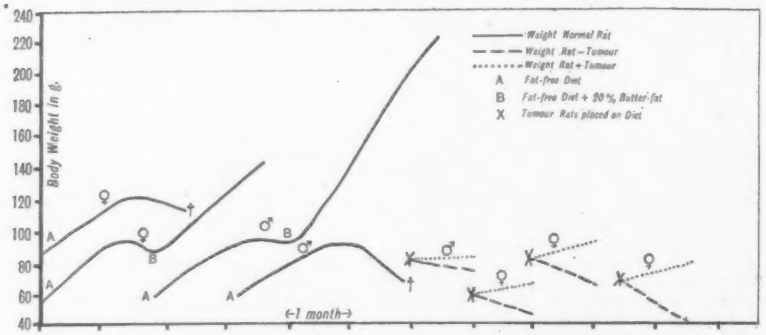

Chart 30. Tumour development, and growth curves of normal and tumour-bearing rats upon diet lacking both fats and the "fat-soluble *A*." Tumour *S*/117*d*. Diet: Ether extracted dried skimmed milk, 50 %, Starch, 50 %.

avian polyneuritis does not occur until the fowl has been fed upon a diet deficient in the so-called "vitamine" for a period of about three weeks. A gradual decrease of body-weight occurs during this period, which apparently corresponds with the period during which many rats show a more or less

successful maintenance of body-weight. The onset of the nerve symptoms in the fowl corresponds roughly with the rapid decline in weight and death of the rat.

Funk [1914, 3] has shown that the period preceding the onset of the nerve disturbances in the pigeon may be shortened by increasing the carbohydrate

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat − tumour g	Change in weight of rat g	Change in weight of host per 1 g tumour
♀						Tumour extensively necrotic and ulcerated.	27·0	105	+27	
♂						ditto	47·0	105	+3·0	
♀						ditto	26·0	55·0	−27·0	
♀						Tumour retrogressed	—	—	—	
♀						ditto	—	—	—	
						Average	33·3	—	+1·0	+0·03

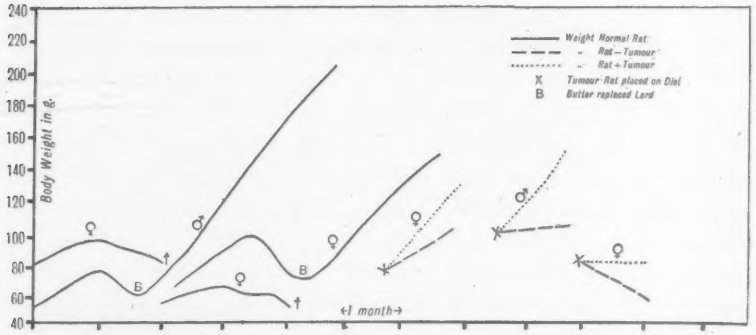

Chart 31. Tumour development, and growth curves of normal and tumour-bearing rats upon diet deficient in "fat-soluble *A*.' Tumour *S*/117*d*. Diet: Ether extracted dried skimmed milk, 50 %, Starch, 30 %, Lard, 20 %.

content of the diet, and he has suggested that a relationship may exist between carbohydrate metabolism and the utilisation of "vitamine." He is inclined to believe that the animal organism possesses a store of the latter substance which may be mobilised for use when a deficiency arises in the diet in this respect.

Whether it is the existence of such a reserve that enables rats to live for some time upon diets deficient in this accessory substance or not is hard to say. Certainly if such is the case the substance is utilised for the purpose of helping to maintain the life of the animal rather than in effecting growth. Tumour growth suffered a slight retardation under these dietary conditions, and was also accompanied by a loss of weight upon the part of the host (Chart 26). Post-mortem examination of these tumour-bearing rats revealed a considerable wastage of skeletal muscle, but the other organs were not visibly affected.

Benedict and Rahe [1917] whose work has already received attention, concluded that the tumour cells are as dependent upon exogenous supplies of this dietary factor, as are the normal cells of the growing animal.

Discussion.

Certain of the points which have arisen during this work have been discussed briefly in the preceding pages, so that the present consideration must be devoted to a more general survey of the results taken as a whole. The outstanding feature which is apparent from a glance at the charts presented with this paper is the comparative failure which has attended the attempts to inhibit the rate of tumour growth by dietary restrictions. In other words it is demonstrated that a considerable degree of tumour growth may occur in hosts fed upon diets which will not permit any normal tissue growth.

The established tumours which formed the subject of this investigation invariably appeared to possess the power of commandeering what part of the food supply of the host they required for their own use. Failing this source of nutrition it was usual to find the tissues of the host sacrificed to no inconsiderable extent in order to provide the requirements of the parasitic cells.

Not altogether unconnected with this subject is the question of tumour growth in pregnant females, a condition where two parasitic cell complexes, each possessing a high growth energy, may be said to be competing for possession of the available food supply.

Clinical observation both on human cases and on experimental animals, has repeatedly demonstrated a retardation of tumour growth during pregnancy, whilst in other instances an increased rate of development upon the part of the neoplasm has been noted under what were apparently identical conditions.

Haaland [1907], Cuénot and Mercier [1909], Uhlenhuth and Weidanz

[1909] are amongst those who have reported a slower rate of tumour growth during pregnancy.

It has been suggested by Jannovics [1912] that the pregnant female presents an unfavourable soil for tumour growth; but Rous [1911, 2] has pointed out that pregnancy under normal conditions is a stimulus to growth and nutrition. The inconsistences in the observations upon the relationship between the degree of tumour growth and pregnancy have been explained by Fichera [cited by Apolant, 1911] as being due to the influence of the number of embryos. Whether this explanation is the correct one, or whether it lies in the relative blood supplies of the tumour and embryos is uncertain, but there is much to support the latter view. It must be remembered that cancer cells perform no function, and that they are therefore free to expend the whole of their energies upon cell division and growth.

The development of the embryo may be for some reasons considered to possess certain parallel features to tumour proliferation, so that it is of interest to note the influence of inadequate dietaries, such as have been studied in this work, upon the former process.

It has been repeatedly observed in this laboratory that animals fed upon inadequate diets will not breed. If females are placed upon the diets soon after they are pregnant the results vary according to the nature of the dietary deficiency.

McCollum, Simmonds and Pitz [1916], found that when the accessory factors *A* and *B* are absent from the diet of the nursing mother, she is unable to rear her young, being only able to supply these substances so long as they are present in her own food supply.

Some results, recently obtained in this laboratory, indicate that in the absence of the water-soluble substance from the diet, growth of the young may continue for a short period after the imposition of the restriction upon the mother; but that sooner or later a rapid decline in the body-weight and health of the young will occur.

Nevertheless, there is unmistakable evidence of growth upon the part of the young for some time after the supply of this dietary factor is cut off from the mother. This result presents similar features to the tumour growth observed under similar conditions.

Returning to a consideration of the process by which tumour cells obtain supplies of food units such as certain amino-acids, when the latter are absent from the diet of the host, it is found that considerable light is thrown upon the subject by a study of certain other conditions.

It is well known that during inanition a transfer of protein "Bausteine"

may occur in the animal organism whereby vital organs, such as the heart, are enabled to exist and functionate as long as possible at the expense of the less important tissues, such as the skeletal muscle. Of particular interest are the classical investigations of Meischer, who described the development of the genital organs of the Rhine salmon at the expense of the musculature, during the period of starvation prior to spawning. In this instance he found that the muscle tissue of the fish might lose 55 % of its weight, without the apparent death of a single muscle cell.

Abderhalden, Bergell and Dörpinghaus [1904] showed that the protein of tissues in inanition possess the same amino-acid distribution as in their normal condition.

This result is confirmed by a few analyses made by the author who could trace no appreciable difference in the amino-acid partition of the tissue proteins of normal chickens, and others whose growth and development had been stunted by dietary inadequacies [Drummond, 1916, 3].

Other examples of a transfer of protein from comparatively unimportant tissues to organs where it is urgently needed are those furnished by the growth of the pregnant uterus and the maintenance of the protein content of the milk of a lactating animal. Both of these occur, as far as possible, at the cost of other tissues, when dietary restrictions necessitate the sacrifice.

The process by which this balance is attained is unknown. It appears however that it is tissues exhibiting at the time a high growth or functionating energy that are protected from the immediate effects of malnutrition by this means. Such for example are the cases which have been given, the uterus during pregnancy, the mammary gland during lactation and the genital organs of the salmon during the spawning period. At the same time it appears probable that all-important organs such as the heart are able to obtain the supplies necessary for their repair and function with equal ease.

Returning to the consideration of the growth of tumours under these circumstances, there seems no reason why the above explanation should not be equally applicable. Tumour cells, particularly those of actively growing sarcomas, possess a high growth potential, as compared with the tissues of the host. At the same time there is no waste of energy in the cancer cell upon functionating processes; cell-division being as far as is known the chief activity exhibited by these units. There is therefore no apparent reason why tumours in possession of an adequate blood supply should not be equally capable of satisfying their requirements by enforcing sacrifice of the tissue of the host, as is the developing embryo.

Any consideration of the results of this investigation must take into account

their bearing upon the treatment of cancer, but little if any hope of alleviating the course of the disease in man can be gathered from the preceding pages. All the results of this experimental research indicate that only the most drastic dietary restrictions, involving a very serious loss of weight upon the part of the host, would have any retarding influence upon tumour growth.

Summary.

1. By imposing certain restrictions upon the diet of a young animal, its growth may be completely inhibited. The present investigation has aimed at a determination of the influence which these dietary inadequacies exert upon the growth of tumours in tumour-bearing subjects.

2. The influence of the following dietary inadequacies upon normal and tumour growth has been studied:

(*a*) Low protein content of the diet.

(*b*) Nitrogen of the diet supplied in the form of a protein possessing a relatively low nutritive value.

(*c*) Absence of certain indispensable amino-acids from the diet.

(*d*) Absence of the equally indispensable accessory growth promoting factors, the "fat-soluble *A*" and the "water-soluble *B*."

3. In the event of a deficiency arising in the diet of the host, the tumour, provided it possesses a satisfactory blood supply, will continue to grow, although the host may be quite unable to do so. There is evidence that this proliferation will proceed at the expense of the tissues of the host, until these are no longer able to supply the missing units.

4. When the host is unable to make good the deficiency, by drawing upon its own reserves, the rate of tumour proliferation will decrease. This occurs at a comparatively early stage when the diet is deficient either in tryptophan or in the water-soluble accessory factor, *B*.

5. There is no evidence that the cells of tumours possess powers of synthetical action which the normal cell of similar type does not possess.

6. It does not appear possible to bring about an inhibition of tumour growth by an employment of dietary restrictions, such as have been used in this research, without the nutritive condition of the host being very seriously impaired.

7. There is, therefore, little hope of bringing about an alleviation of the disease in man by the imposition of dietary restrictions, such as are described in this communication.

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